Hiroshima J. Med. Sci.
Vol.38, No.3, 125～129, September, 1989
HIJM 38-21

Effects of a Diphenyl Ether Herbicide (CNP Emulsion) on Mouse Fetuses

Yukio HIRAOKA, Atsushi IMADA, Junko TANAKA and Hisanori OKUDA

Department of Hygiene, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan

ABSTRACT

We investigated the effects of a diphenyl ether herbicide, a chlornitrophen (CNP) emulsion, on mouse fetuses.

An MO emulsion was used as the experimental chemical twenty percent of this herbicide consisting of CNP. CNP and other components were extracted and evaporated (undegraded solution). The chemical was diluted 10 times by deionized water and then exposed to sunlight until the CNP concentration in the mixture became 10% of the initial CNP concentration. CNP, degradation products, and other materials were extracted from the mixture and evaporated (degraded solution).

The undegraded and degraded solutions were administered subcutaneously to the backs of pregnant mice from the 6th to 15th fetal day, once daily. The mice were sacrificed on the 18th day of pregnancy. The number of fetuses and implantations, their weights, externals and skeletons were observed. Infant mice born from maternal mice treated in the same way as above were weighed once a week.

Mean fetal body weights of the undegraded and degraded solution dose groups were significantly lower than those of the control ($p<0.001$). Fetal skeletal abnormalities were also higher than those of the control. The degraded solution dose group differed particularly in this regard from the control ($p<0.05$). In the degraded solution dose group, all 21 newborn infants from two maternal mice out of a total of 43 newborn infants from four maternal mice died within one week.

The above results suggest that the degraded solution of CNP is no less noxious on mouse fetuses than the undegraded solution. Thus, more consideration should be given not only to the original chemicals but to the degradation products as well when assessing agricultural control chemicals for their safety.

Key words: *Diphenyl ether herbicide, Mouse fetuses*

Diphenyl ether herbicides are used in large quantities from May to June as early herbicides for paddy fields, before and after rice-planting. They are then released into rivers[3,6,8].

It is reported that aquatic organisms in rivers accumulate large amounts of herbicides[9,13] with marked bioconcentration of chlornitrophen (CNP). The bioconcentration of CNP of *Carassius auratus* and *Ribolodon hakonesis* is around 1600 times[4] and that of *Corbicula japonica*, around 2000 times[8]. Thus, people may take in CNP by eating fish and shellfish to an extent which is hazardous to their health.

Nakao has carried out a detailed hemological and histopathological investigation to detect acute, subacute, and chronic toxicity of CNP in the mouse, rat, and rabbit and found very low toxicity[7]. However, the effects of CNP on embryo, a most sensitive stage to noxious materials, was not examined in that paper. Furthermore, despite extensive reference research, we were unable to find any previous report in this regard.

Thus, in the present study, the effects of CNP on mouse fetus were investigated.

MATERIALS AND METHODS

1. Experimental chemicals: An MO emulsion made by Sankyo Co., Ltd. (20% CNP and 80% organic solvent, emulsifier, and other constituents), a diphenyl ether herbicide quite frequently used in Japan, was used in the present study.

1). Preparation of the undegraded solution: The MO emulsion diluted with an equal amount of deionized water was mixed with n-hexane and shaken vigorously. The n-hexane layer was collected and evaporated.

2). Preparation of the degraded solution: The MO emulsion diluted 10 times with deionized water was put into a stainless container (63 × 42 cm) and exposed to sunlight. The CNP concentration in the

mixture was checked regularly by gas chromatography with a flame ionization detector (GC-FID). When about 90% of the CNP had degraded, exposure was discontinued. This mixture was mixed with n-hexane and shaken vigorously. The n-hexane layer was collected and evaporated.

An aliquot of the degraded solution was sent to Kyoto Analysis Center of Shimazdu Seisakusho Co., Ltd. and qualitative analysis was carried out with a gas chromatograph-mass spectrometer.

2. The effects of the undegraded and degraded solutions on mouse fetuses: Specific pathogen-free male and female mice of ICR strain were received at 8 weeks of age and reared more than 1 week in our laboratory. Food and water were given *ad libitum*. The 1st day of pregnancy was considered to start 9 A.M. on the following morning when vaginal plugs were found and randomly divided into groups. An olive oil-acetone (3:1) mixture was used to dilute the undegraded solution and physiological saline to dilute the degraded solution. The diluter was different for the two solutions so as to obtain the best conditions for mixing. The olive oil-acetone mixture (control 1), physiological saline (control 2), the undegraded solution and the degraded solution were administered subcutaneously to the backs of pregnant mice from the 6th to 15th day of pregnancy, once daily. The dosage was 0.05 ml/10g body weight (BW) for the undegraded solution and 0.1 ml/10g BW for the degraded solution, each containing 1 g/kg BW of CNP. The mice were sacrificed on the 18th day of pregnancy and the number of fetuses and implantations, weights of fetuses, and externals were observed visually. The fetuses were stained by Inouye's method[2] and observed by stereoscopic microscopy.

3. Growth of newborn infants from maternal mice administered with the undegraded or degraded solution: Specific pathogen-free male and female mice of the ICR strain were received at 8 weeks of age and reared for more than 1 week at our laboratory. Females which underwent pregnancy, birth, and nursing were used in this experiment. The undegraded solution, the degraded solution, control 1, and control 2 were administered to the pregnant mice in the same way as in experiment 2. The newborn infants born naturally ware reared carefully and their weights were measured once a week.

4. Measurement of CNP: An aliquot of a sample was mixed with n-hexane, shaken vigorously, and centrifuged at 2000 rpm for ten minutes. 1 μl of n-hexane was used for analysis. GC conditions for the analysis were as follows: Gas chromatograph: Shimazdu GC14A equipped with flame ionization detector, Column: CBP-1 (length 15m, ID 0.53 mm, film thickness 3.0μm), Column temperature: 180°C, Injector and detector temperature: 230°C, Carrier gas: Helium (0.25 kg/cm^2). The CNP standard used for chemical analysis was obtained from Wako Pure Chemical Industries Ltd. Tokyo and 99% pure.

RESULTS

1. Effects of the undegraded and degraded solutions on fetuses (Table 1): The mean fetal body weight of control 1, the undegraded solutions, control 2, and the degraded solutions were 1.36g, 1.18g, 1.34g, and 1.18g, respectively. The mean fetal body weights of the undegraded and degraded solutions were significantly lower than the control ($p<0.001$). However, there was no significant

Table 1. Effects of Undegraded Chlornitrophen (CNP) Emulsion and Degraded CNP Solution on Pregnant Mice

	Control 1	Undegraded CNP solution	Control 2	Degraded CNP solution
Dosage of CNP (g/kgBW)	0	1	0	1
No. of tested mice with plugs	7	9	8	9
No. of live mice (A)	7	9	8	9
No. of mice with live fetuses (B)	7	8	8	7
Successful pregnancy rate (B/A)	100	89	100	78
Total no. of implants	36	38	41	42
Total no. of live fetuses	36	36	39	39
No. of live fetuses per litter	12.0	12.0	13.0	13.0
No. (%) of resorbed or dead fetuses	0	2 (5.3)	2 (4.9)	3 (7.1)
Mean fetal body weight (g; M ± SD)	1.36 ± 0.08	1.18 ± 0.06***	1.34 ± 0.09	1.18 ± 0.11***
No. (%) of live fetuses with external abnormalities	1	1	0	0
No. (%) of live fetuses with skeletal abnormalities	0	3 (8.3)	0	6 (15*)

***: $p<0.001$, compared to the corresponding control. Statistical analysis performed by means of the students' t test. @
*: $p<0.05$, compared to the control 2. Statistical analysis employed Fisher's direct probability method.
Control 1: Olive oil + acetone. Control 2: Physiological saline.

@; It was confirmed independently by Burtlett's method that there was no significant difference in variance among body weights of fetuses from each maternal mouse in controls 1, 2, the undegraded and degraded solutions was noted before the students' t-test was carried out.

Table 2. Growth of New-born Infants

	0 wk		1st wk		2nd wk		3rd wk		4th wk		5th wk	
	No.	Mean ± S.D.	No.	Mean ± S.D.	No.	Mean ± S.D.	No.	Mean ± S.D.	No.	Mean ± S.D.	No.	Mean ± S.D.
Control 1												
Sample 1	13	1.48 ± 0.10	13	4.00 ± 0.22	13	6.43 ± 0.51	13	7.74 ± 0.63	13	16.6 ± 1.07	13	23.8 ± 1.92
2	11	1.36 ± 0.06	11	4.52 ± 0.19	11	7.59 ± 0.40	11	11.6 ± 0.40	11	19.6 ± 1.24	11	25.2 ± 1.82
3	15	1.42 ± 0.07	15	4.17 ± 0.41	15	6.51 ± 0.64	15	10.0 ± 1.21	15	16.2 ± 1.77	15	24.1 ± 2.62
4	13	1.42 ± 0.05	13	4.10 ± 0.24	13	6.35 ± 0.36	13	9.68 ± 0.87	13	16.0 ± 1.84	13	21.8 ± 2.72
CNP												
Sample 1	14	1.46 ± 0.07	14	4.10 ± 0.34	14	5.71 ± 0.55	14	9.04 ± 1.17	14	15.4 ± 1.67	14	20.5 ± 2.38
2	10	1.65 ± 0.13	10	5.00 ± 0.28	10	7.29 ± 0.27	10	11.4 ± 0.57	10	18.8 ± 1.75	10	24.4 ± 2.84
3	14	1.32 ± 0.09	12	4.24 ± 0.20	12	6.73 ± 0.29	12	9.96 ± 0.60	12	16.6 ± 1.31	12	21.9 ± 2.65
4	13	1.52 ± 0.08	13	4.12 ± 0.33	13	6.57 ± 0.49	13	10.5 ± 1.06	13	18.1 ± 2.16	13	23.7 ± 3.14
5	14	1.53 ± 0.05	14	3.39 ± 0.23	14	5.46 ± 0.30	14	8.53 ± 0.72	14	15.6 ± 1.09	14	22.4 ± 1.38
Control 2												
Sample 1	15	1.30 ± 0.06	15	3.36 ± 0.31	15	4.93 ± 0.61	15	5.70 ± 0.90	15	12.1 ± 2.33	15	18.4 ± 3.06
2	14	1.29 ± 0.10	7	3.98 ± 0.19	7	7.56 ± 0.32	7	10.8 ± 0.47	7	18.9 ± 1.71	7	24.3 ± 2.15
3	13	1.39 ± 0.12	13	3.09 ± 0.47	12	5.99 ± 0.66	12	8.69 ± 1.39	12	15.5 ± 2.49	12	20.8 ± 2.65
4	15	1.45 ± 0.07	12	2.46 ± 0.36	12	5.34 ± 0.59	12	6.92 ± 0.82	12	14.4 ± 1.84	12	21.6 ± 2.46
5	11	1.55 ± 0.10	11	3.19 ± 0.31	11	5.98 ± 0.28	11	8.47 ± 0.62	11	15.7 ± 1.47	11	23.0 ± 2.41
Degraded CNP												
Sample 1	11	1.01 ± 0.12	9	3.33 ± 0.43	8	6.80 ± 0.62	8	9.30 ± 0.88	8	16.5 ± 1.99	8	21.9 ± 3.38
2	7	1.05 ± 0.10	0									
3	11	1.43 ± 0.08	11	2.93 ± 0.19	11	5.42 ± 0.17	11	6.19 ± 0.43	11	11.7 ± 1.34	11	18.4 ± 2.00
4	14	1.18 ± 0.09	0									

Control 1 : Olive oil + acetone. Control 2 : Physiological saline.
"No." indicates the number of new-born infants.

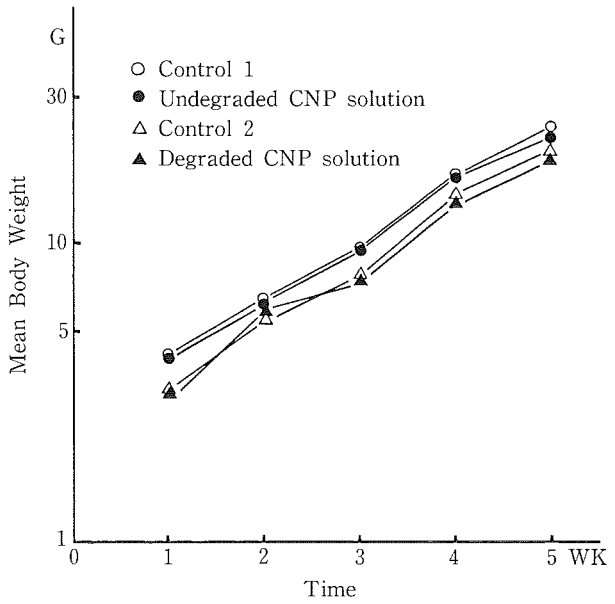

Fig. 1. Growth of infants

difference between the undegraded and degraded solutions.

No skeletal abnormality was observed in the fetuses of controls 1 and 2, but the incidence of skeletal abnormality was 8.3% in the undegraded solution and 15% in the degraded solution. There was a significant difference between the degraded solution and control 2 ($p<0.05$). Specific skeletal abnormalities observed frequently included a small round cavity in the lower sternum and absence of cartilage in the tip of rib.

Regarding the rate of maternal death, the successful pregnancy rate, and the rate of resorbed or death fetuses, there were little differences between the undegraded solution and control 1 and between the degraded solution and control 2.

2. Growth of newborn infants (Table 2): With the degraded solution, all 21 newborn infants from two maternal mice out of a total of 43 newborn infants from four maternal mice died within one week. However, the surviving newborn infants, growth was essentially the same level as that of control 2. In controls 1 and 2 and the undegraded solution, some newborn infants died, but not all newborn infants died within a week in the experimental groups.

Changes in the mean body weights of mice in each experimental group are shown in Figure 1. The mean body weight of the undegraded solution was somewhat less than that of control 1 from the 1st week through 5th week. That of the degraded solution was somewhat less than that of control 2 at the 1st, 3rd, 4th, and 5th weeks.

DISCUSSION

Generally, acute toxicity of CNP is reported to be very low and LD50 values in the mouse are 11800 mg/kg and 4500 mg/kg transcutaneously and transabdominally, respectively[10]. In the present experiment, the dosage of CNP to pregnant mouse was 1000 mg/kg and none of the animals died during the experiment. The successful pregnancy rate and the rate of dead fetuses in the undegraded and degraded solutions were not different from the respective values of the control. However, differences in the mean fetal body weights between the undegraded and degraded solutions and the control were remarkable. There was no difference between the undegraded solution and degraded solutions.

Each dosage contained 1 g/kg of CNP for both the undegraded and degraded solutions, and the rest of the dosage consisted of some kinds of organic solvents in the case of the undegraded solution. Degradation products of CNP such as CNP-NH2, trichlorphenol were also included in the degraded solution. The mean fetal body weights in the undegraded and degraded solutions were less than those of the control. Possibly a combination of CNP, its degradation products, and organic solvents had deleterious effects on various mice, resulting in evident malnutrition.

With regard to skeletal abnormalities, the incidence with the undegraded and degraded solutions was higher than in the control. With the degraded solution, it was higher than with the undegraded solution. Degradation products of CNP are thus considered to cause skeletal abnormalities in the mouse fetus.

The mean body weights with the undegraded and degraded solutions tended to be somewhat less than those of the control. With the degraded solution, all infants from two maternal mice out of four died within 1 week. Thus the degraded solution containing degradation products caused not only reduction in mean fetal body weight but also death of the newborn infants. The effects of these chemicals did not, however, persist: during 5 weeks of observation, no effects on surviving infants were noted.

Hiraoka et al[1] carried out an investigation with fenitrothion (MEP), an organic phosphorous pesticide, by the same procedure as that employed in this study. On comparing the results with CNP and MEP, we noted that the mean fetal body weights of CNP and MEP dose groups tended to be smaller than the control. With respect to skeletal abnormalities, there was little difference in the incidence between the MEP dose group and control, but the incidence in CNP dose group was higher than in the control.

The results of the present experiment do not reflect actual environmental problems since the dosage, 1000 mg/kg, was extremely high. However, CNP is a chemical of high accumulation and CNP-NH2, a reductive metabolite of CNP, has the mutagenicity[12] and the formation ability of methemoglobin[5]. CNP easily forms CNP-NH2 by a process of reduction in nature[11]. Thus, more careful attention should be directed to CNP and its

derivatives.

ACKNOWLEDGEMENT

This study was supported by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Science, and Culture of Japan (63570238).

(Received May 31, 1989)
(Accepted July 10, 1989)

REFERENCES

1. **Hiraoka, Y., Imada, A., Tanaka, J. and Okuda, H.** 1989. The toxicity of organic phosphorous pesticide, fenitrothion emulsion, and its degraded solution on mice. (Submitted to Environ. Pol.).
2. **Inouye, M.** 1976. Differential staining of cartilage and bone in fetal mouse skeleton by alcian blue and alizarin red S. Cong. Anom. **16**: 171–173.
3. **Mikuriya, H. and Miyahara, K.** 1983. Pollution of creek waters in Saga Pre. by pesticides applied in paddy fields. Ecological Chemistry **6**: 23–33.
4. **Mishima, Y., Kato, Y., Seki, T., Tsunoda, A., Suzuki, T., Sasaki, M. and Oba, M.** 1987. Environmental pollution by diphenyl ether herbicides in Sendai city. J. Environ. Contam. Cont. **23**: 32–38.
5. **Miyauchi, M., Koizumi, M. and Uematsu, T.** 1981. Studies on the toxicity of chlorinated p-nitrobiphenyl ether. I-Methemoglobin formation in vitro and in vivo induced by nitroso and derivatives of chlorinated biphenyl ether. Biochem. Pharmacol. **30**: 3341–3346.
6. **Nakaminami, G., Ishida, N., Kunimatsu, T. and Tsuruyama, S.** 1985. The pollution of Lake Biwa, Yodo river, Chikugo river, Ariake sea and others by diphenyl ether herbicides. Ecological Chemistry **8**: 3–11.
7. **Nakao, A.** 1975. An experimental study on the toxicity of 2,4,6-trichlorophenyl-4'-nitrophenyl ether (CNP). Fukuoka Acta. Medica **66**: 123–153.
8. **Ohyama, T., Jin, K., Katoh, Y., Chiba, Y. and Inouye, K.** 1987. Fate and behavior of herbicides, Butachlor, CNP, Chlomethoxyline in river water, Shellfish, and sediments of the Ishikari river. Bull. Environ. Contam. Toxicol. **39**: 555–562.
9. **Ono, H. and Nakanishi, J.** 1987. Herbicide, CNP contamination in the Lake Kasumigaura basin. Wat. Res. **21**: 669–675.
10. **Shitatori, H. and Sukitsuka, S.** 1975. The fate of degradation products in soil. Abstracts of 26th conference of agricultural biochemistry. 50.
11. **Tohyama, T. and Takazawa, Y.** 1971. CNP (MO). Technology of Pesticides Products. **23**: 1–14.
12. **Watanabe, M., Yoshioka, T., Sawaguchi, T., Miyauchi, M. and Uematsu, T.** 1983. Studies on the toxicity of chlorinated phenyl nitrophenyl ethers and their possible metabolites. Abstracts of the 10th Conference on Pollutants in the Environment and Their toxicology. 29–31.
13. **Yamagishi, T. and Akiyama, K.** 1981. 1,3,5-trichloro-2-(4-nitrophenoxy) benzen in fish, shellfish, and sea water in Tokyo bay, 1977—1979. Arch. Environ. Contam. Toxicol. **10**: 627–635.