Bull. Environ. Contam. Toxicol. (1991) 47:689–695

Bulletin of Environmental Contamination and Toxicology

Toxicokinetics of Methylmercury and Mercuric Chloride in Mouse Embryos *In Vitro*

Ichiro Naruse,[1] Nobuo Matsumoto,[2] and Yuji Kajiwara[1]

[1]Pathology Section, National Institute for Minamata Disease, Minamata, Kumamoto 867, Japan, and [2]Department of Hygiene, Tokyo Jikeikai Medical College, NishiSinbashi, Minato-ku, Tokyo 105, Japan

Severe Fetal Minamata Disease leads to cerebral palsy, blindness, deafness accompanying microcephaly, and makes it impossible to stand, walk or talk, (WHO 1990). These severely affected children have been considered to be exposed to methylmercury (MM) during mid and late gestation periods. However, it is very rare to find gross malformations in human infants. From this knowledge, we speculated that the exposure to MM during early gestation period must induce fetal death, though we have no epidemiological data.

Fetotoxicity and teratogenicity of MM have been confirmed in experimental studies in mice, hamsters and rats (Spyker and Smithberg 1972; Harris et al. 1972; Inouye and Murakami 1975). Pre- and early postimplantation embryonic losses induced by MM were also recorded in mice (Verschaeve and Leonard 1984). It has been difficult to study the dying processes of mouse embryos during organogenesis induced in utero by some agents. Whole embryo culture has alleviated some of the difficulties. This technique must be a good method to investigate the embryolethality.

Inorganic mercury such as MC is poorly absorbed from the gastrointestinal tract (Berlin 1986) and transfers inefficiently to the fetus but accumulates in the placenta in the mid and late gestation period (Suzuki et al.1967; Gale and Hanlon 1976; Holt and Webb 1986). Therefore, it has been suggested that MM has much greater toxicity than MC in general. But, toxicity of MC shows another aspect during very early gestational stages, especially before the formation of the placenta. That is, female mice treated with MC showed abnormal preimplantation embryos with lower doses than those of MM (Kajiwara and Inouye 1986). In their experiment, maternal factors were included, that is, mother mice showed very severe intoxication. Using the whole embryo culture system, such maternal factors can be excluded. In the present experiment, we examined the embryotoxicity differences between MM and MC in vitro, and then

Send reprint requests to I. Naruse at the above address.
2: passed away on May 5, 1990.

discussed those results using the data of transfer of MM and MC into the embryo.

MATERIALS AND METHODS

Two Slc:ICR female mice were mated with 2 males of same colony overnight. Pregnancy was dated as day 0 if a vaginal plug was found in the next morning. On day 8.5 of pregnancy, females were killed by cervical dislocation. The method employed for the in vitro cultivation of mouse embryos is described previously (Tsutsui and Naruse 1987; Naruse et al. 1988). Uteri were removed and placed in a 35-mm plastic culture dish containing sterile Hank's balanced salt solution. Decidua and Reichert's membrane were removed under a dissecting microscope using watchmaker's forcepts. Embryos with visceral yolk sac and ectoplacental cone intact were then transfered to the rotator whole embryo culture system (New and Cockroft 1979). The culture bottles were gassed with a mixture of $5\%O_2/5\%CO_2/90\%N_2$ continuously at $37^{\circ}C$ and rotated at 30 rpm. Each bottle contained 3 ml immediately centrifuged rat serum (Steel and New 1974) to which was added 50 IU/ml penicillin G and 50 µg/ml of streptomycin after heat inactivation ($56^{\circ}C$, 30 minutes). Three embryos were cultured together in each bottle. Gas was changed to $20\%O_2/5\%CO_2/75\%N_2$ at 16 hours, and the medium was changed at 24 hours. Volumes of 20-100 µl of MM diluted with distilled water were added to 3 ml of culture serum. The embryos were treated with 20-100 µM of MM for 48 hours. Volumes of 15-60 µl of MC diluted with distilled water were added to 3 ml of culture serum. The embryos were treated with 50-200 µM for 48 hours. After cultivation for 24 hours, heart beat and yolk sac expansion were checked. Heart beat and blood circulation on the yolk sac were checked and the yolk sac diameter and crown-rump length were measured on a section paper under the dissection microscope at 48 hours. After rinsing with Hank's solution, they were fixed in Bouin's solution. The status of embryonic axial rotation, abnormalities, and degree of differentiation were recorded and somite pairs were counted in the fixed embryos. Yolk sac diameter, crown-rump length, and somite counts were analyzed by t-test. Frequency data, such as incidence of specific defects, were analyzed pairwise using Fisher's test for uncorrelated proportions.

1.5×10^{-2} µCi of $[^{203}Hg]HgCl_2$ /ml (medium) or 5.2×10^{-2} µCi of $[^{203}Hg]CH_3HgCl$ /ml(medium) were added to the culture medium at 20 hours of cultivation, and three embryos were sampled at 2, 4, 8, and 24 hours after the addition of radioisotope. The transfer into the whole embryo including membrane was measured by γ-counter (Aloka Auto Well Gamma System ARC-600) after rinsing with saline twice. Counts of embryo proper were measured after taking off the yolk sac membrane and ectoplacental cone and rinsing with saline twice.

RESULTS and DISCUSSION

When treated with 20 μM of MM, embryos were almost the same as those of the control group. In the group treated with 30 μM, the number of embryos which showed a beating heart and blood circulation on the yolk sac at 48 hours was decreased, and the somite number was also decreased. In the group treated with 50 μM, the embryos showed some alterations in heart beat, axial rotation, yolk sac diameter, crown-rump (C-R) length, number of somites (Table 1). They also showed some abnormalities such as growth retardation, hypoplasia of hindlimb and stunted head. In

Table 1. Effects of methylmercury on the development of mouse embryos in vitro

	Heart beat at 24h	Heart beat at 48h	Axial rotation	Blood circulation	Yolk sac diameter (mm±SD)	C-R length (mm±SD)	No. of somites (No.±SD)
Control	15/15	15/15	15/15	14/15	4.6±0.4	4.2±0.3	30±3.1
20 μM	12/12	11/12	11/12	10/12	4.3±0.6	4.0±0.3	28±3.4
30 μM	12/12	5/12**	10/12	3/12**	4.8±0.4	3.9±0.3	27±4.3*
50 μM	11/12	5/12**	5/12**	2/12**	4.0±0.8**	3.1±0.5**	21±7.7**
100 μM	1/12**	0/12**	0/12**	0/12**	1.3±0.3**	-	-

* $p<0.05$, t- or Fisher's tests for dose comparison with control.
**$p<0.01$, t- or Fisher's tests for dose comparison with control.

Table 2. Abnormalities of the mouse embryo induced by methylmercury in vitro

	Growth retardation	Neural tube closure defect	Hypoplasia of hindlimb	Edema	Tail anomalies	Stunted head	Hypoplasia of eye
Control	0/15	0/15	1/15	0/15	0/15	0/15	0/15
20 μM	0/12	0/12	0/12	0/12	1/12	0/12	0/12
30 μM	3/12	0/12	4/12	0/12	1/12	2/12	2/12
50 μM	6/12**	2/12	8/12**	3/12	0/12	5/12**	4/12
100 μM	-	-	-	-	-	-	-

**$p<0.01$, Fisher's test for dose comparison with control.

the group treated with 100 μM of MM, most embryos died by 24 hours after exposure, and all the embryos were dead by 48 hours (Table 2). The embryo toxicity of MM was quite variable. For example, in the 50 μM group, some embryos showed severe defects, such as growth retardation, neural tube closure defects, hypoplasia of fore- and hindlimbs and eyes, edema, and stunted head, however, the other embryos developed almost normally. 100 μM was a lethal dose.

The embryos treated with 50 μM of MC showed defects in blood circulation on the yolk sac, crown-rump length, and number of somites, but did not show abnormalities. 100 μM of MC induced a decrease of yolk sac diameter, crown-rump length, and number of somites, but did not cause abnormalities. Some embryos treated with 200 μM of MC died by 24 hours, but others survived and showed many alterations such as no blood circulation on the

Table 3. Effects of mercuric chloride on the development of mouse embryos in vitro

	Heart beat at 24h	Heart beat at 48h	Axial rotation	Blood circulation	Yolk sac diameter (mm±SD)	C-R length (mm±SD)	No. of somites (No.±SD)
Control	15/15	15/15	15/15	14/15	4.6±0.4	4.2±0.3	30±3.1
50 μM	12/12	10/12	9/12	5/12**	4.3±0.5	3.6±0.8*	23±10.3*
100 μM	12/12	12/12	10/12	9/12	4.1±0.4**	3.6±0.6**	24±7.2**
200 μM	5/12**	0/12**	8/12	0/12**	3.0±1.0**	2.5±0.7**	16±5.0**

* $p<0.05$, t- or Fisher's tests for dose comparison with control.
**$p<0.01$, t- or Fisher's tests for dose comparison with control.

Table 4. Abnormalities of the mouse embryos induced by mercuric chloride in vitro

	Growth retardation	Neural tube closure defect	Hypoplasia of hindlimb	Edema	Tail anomalies	Stunted head	Hypoplasia of eye
Control	0/15	0/15	1/15	0/15	0/15	0/15	0/15
50 μM	3/12	0/12	2/12	1/12	4/12	4/12	1/12
100 μM	1/12	0/12	5/12	1/12	1/12	3/12	0/12
200 μM	4/6**	2/6	4/6**	4/6**	6/6**	2/4	4/6**

**$p<0.01$, Fisher's test for dose comparison with control.

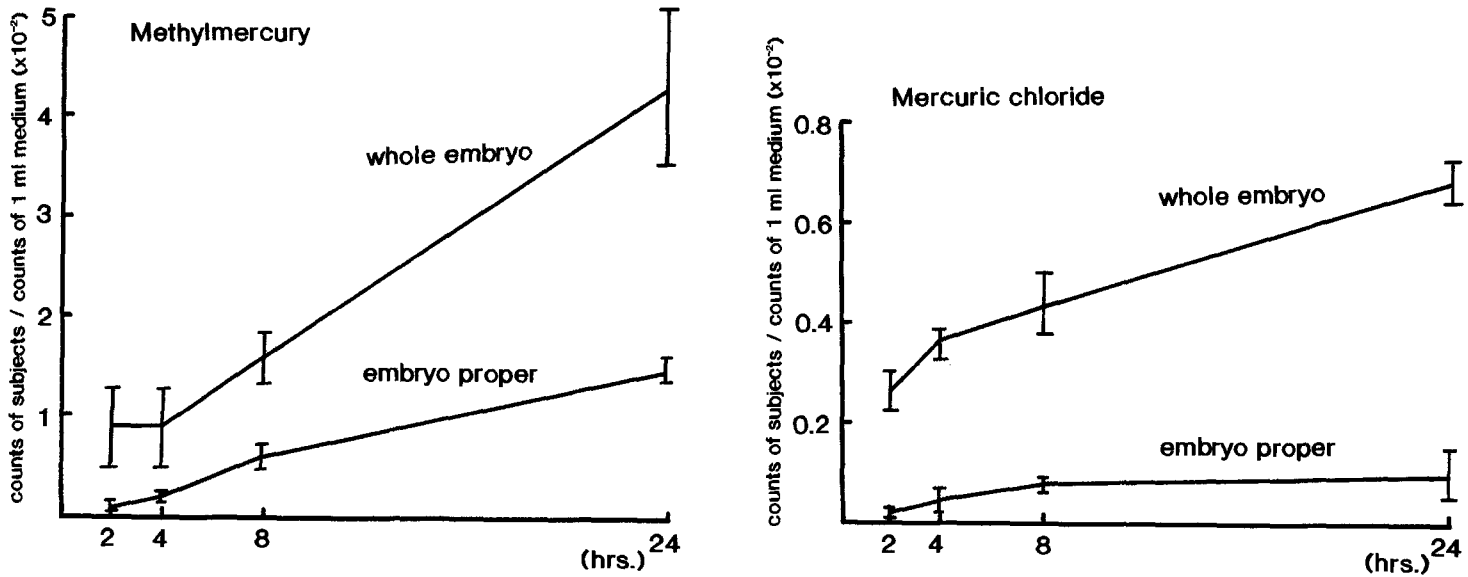

Figure 1. Transfer of methylmercury and mercuric chloride into the embryo in vitro. The graphs were shown as the means of counts of subjects / counts of 1 ml medium ($x10^{-2}$) including SD.

yolk sac, decreased yolk sac diameter, crown-rump length, and number of somites and showed growth retardation, hypoplasia of hindlimb, edema and hypoplasia of the eye (Table 3, 4). The transparency of the yolk sac membrane was decreased after exposure to more than 50 μM of MC and blood circulation on the yolk sac was defective. The crown-rump length and number of somites were also decreased in these groups. These results suggest that the embryotoxicity of MM, especially embryolethality, is more than 2 times greater than that of MC.

Fig. 1 shows the transfer of MM and MC into the mouse embryo in vitro. The concentrations of MM in whole embryo and embryo proper were much higher than those of MC. The possible mechanisms of MM embryotoxicity have been reviewed by Miura and Imura (1987). MM readily crosses the placenta and accumulates in the fetus; accelerated accumulation occurs at later pregnant stages. MC is however trapped in the yolk sac of rat and mouse embryos during late gestational stages (Ohsawa et al. 1981; Inouye 1989). This mechanism was supported even in the organogenesis stage of the mouse embryo cultured in vitro in the present experiment.

Another problem is the mechanism of the penetration differences of MM and MC through the yolk sac membrane. The transfer of MC into the whole embryo and embryo proper was much lower than those of MM as shown in Fig. 1. In the group treated with MC, the transparency of the yolk sac membrane decreased and blood circulation on the yolk sac was defective after exposure to low doses of MC. These data indicated that the yolk sac can protect the embryo from MC exposure, but not from MM exposure. The cause of embryotoxicity of MC may be from the defects of yolk sac. The mechanism of the penetration differences of MM and MC through the plasma membrane is still unknown. It has been reported that MM easily penetrate the plasma membrane than MC (Nakada et al. 1980). While, MC show a high affinity to lipids suggesting high affinity to phospholipids in the plasma membrane, but MM is almost inert to these lipids (Nakada and Imura 1983). The data in the present experiment cannot be explained by the reaction with phospholipids in the plasma membrane. Other alternative mechanism such as energy-dependent transport may explain this data. After invasion of mercury into the embryo proper, the corrosive action of mercury can damage any tissue, probably attacking the microtubules (WHO 1976; 1990).

Acknowledgments. The authors express their hearty thanks to Prof. Scott WJ Jr., Children's Hospital Research Foundation, University of Cincinnati, for the review and revision of the manuscript, and to Miss Arakawa for the assistance in the experiment.

REFERENCES

Berlin M (1986) Mercury. In: Friberg L, Nordberg GF, Vouk VB

(eds.) Handbook on the toxicology of metals. Vol. II. Specific Metals. Elsevier, New York, pp 387-445
Gale TF, Hanlon DP (1976) The permeability of the Syrian hamster placenta to mercury. Environ Res 12: 26-31
Harris SB, Wilson JB, Printz RH (1972) Embryotoxicity of methylmercuric chloride in golden hamster. Teratology 6:139-142
Holt D, Webb M (1986) The toxicity and teratogenicity of mercuric mercury in the pregnant rat. Arch Toxicol 58:243-248
Inouye M, Murakami U (1975) Teratogenic effect of orally administered methylmercuric chloride in rats and mice. Cong Anom 15:1-9
Inouye M (1989) Teratogy of heavy metals: Mercury and other contaminants. Cong Anom 29:333-344
Kajiwara Y, Inouye M (1986) Effects of methylmercury and mercuric chloride on preimplantation mouse embryos in vivo. Teratology 33:231-237
Miura K, Imura N (1987) Mechanism of methylmercury cytotoxicity. CRC Critical Review Toxicol 18:215-243
New DAT, Cockroft DL (1979) A rotating bottle culture method with continuous replacement of the gas phase. Experimentia 35:138-140
Nakada S, Imura N (1983) Susceptibility of lipids to mercurials. J Appl Toxicol 3:131-134
Nakada S, Nomoto A, Imura N. (1980) Effect of methylmercury and inorganic mercury on protein synthsis in mammalian cells. Ecotoxicol Environ Safety 4:184-190
Naruse I, Collins MD, Scott WJ (1988) Strain differences in the teratogenicity induced by sodium valproate in cultured mouse embryos. Teratology 38: 87-96
Ohsawa M, Fukuda K, Kawai K (1981) Accelerated accumulation of methylmercury in the rat fetus at the late pregnant stage. Ind Health 19:219-221
Spyker JM, Smithberg M (1972) Effects of methylmercury on prenatal development in mice.Teratology 5:181-190
Steel CE, New DAT (1974) Serum variants causing the formation of double hearts and other abnormalities in explanted rat embryos. J Embryol Exp Morph 31:707-719
Suzuki T, Matsumoto N, Miyata T, Katsunuma H (1967) Placental transfer of mercuric chloride, phenyl mercury acetate and methyl mercury acetate in mice. Ind Health 5: 149-155
Tsutsui Y, Naruse I (1987) Murine cytomegalovirus infection of cultured mouse embryos. Am J Path 127: 262-270
Verschaeve L, Leonard A (1984) Dominant lethal test in female mice treated with methylmercury chloride. Mutat Res 136:131-136
WHO (1976) Environmental Health Criteria I: Mercury, Geneva, World Health Organization.
WHO (1990) Environmental Health Criteria 101: Methylmercury, Geneva, World Health Organization.

Received March 10, 1991; accepted June 5, 1991.