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Studies on Ergothioneine. VIII.[1] Preventive Effects of Ergothioneine on Cadmium-induced Teratogenesis

Tadanori Mayumi,[a] Kyoko Okamoto,[a] Kaoru Yoshida,[a] Yuichi Kawai,[a] Hiroko Kawano,[a] Takao Hama,*,[a] and Keiichi Tanaka[b]

Faculty of Pharmaceutical Sciences,[a] and Faculty of Nutrition,[b] Kobe-Gakuin University, Arise, Igawadani-cho, Tarumi-ku, Kobe, 673, Japan

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The possible preventive effects of ergothioneine on cadmium-induced teratogenesis were examined. Cd (2 mg/kg) was administered *i.p.* to mice on day 7 of pregnancy. The treatment group of mice was given *i.p.* ergothioneine daily at a dose of 80 mg/kg during days 5 to 9, whereas the control group was given saline solution. These mice were subjected to Cesarean section on day 17 of gestation, and the number of dead fetuses, the body weight of living fetuses, and external, skeletal and visceral abnormalities were recorded. On the other hand, ^{109}Cd (50 μCi/2 mg Cd/kg) was administered *i.p.* to other pregnant mice on day 7, and the tissue distribution of the Cd was examined on days 8 and 17 of gestation.

The results of the series of experiments were as follows.

(1) Administration of ergothioneine alone had no effect on external, skeletal and visceral abnormalities.

(2) Of 257 cases, fetuses in 79 cases died or were resorbed after Cd administration. The body weight of living fetuses was significantly reduced, and exencephalia (64%), open eye (31.5%) and marked skeletal malformations of cervical, thoracic and lumbar vertebrae were observed.

(3) Ergothioneine very significantly reduced the incidences of exencephalia, open eye and skeletal abnormalities caused by Cd.

(4) There were no differences between both groups in the *in vivo* distribution of ^{109}Cd, or in the form of ^{109}Cd present in the liver, kidney and placenta.

These results suggest that ergothioneine inhibits Cd-induced teratogenesis through a mechanism other than causing changes in the maternal distribution of Cd and in Cd transfer to fetuses.

Keywords——ergothioneine; preventive effects on teratogenesis; teratogenesis; cadmium; pregnancy; exencephalia; skeletal malformation; dead and resorbed fetuses; distribution of ^{109}Cd in rat

In studies aimed at determining the physiological significance of ergothioneine (2-mercaptohistidine trimethyl betaine; Erg.), we have succeeded in establishing a method for determination of Erg. in biological samples by high performance liquid chromatography (HPLC) and reported its application for metabolic studies.[2] We further examined the binding form of Erg. *in vivo*[3] and its effects on red blood cells,[4] *etc.* The results suggested that Erg., which is taken up from the diet and contained in large amounts in the liver, exists partially in a form bound to proteins of high molecular weight, but largely in the free form (4.8 mg/100 g liver), and takes part in a redox system involving its –SH group as in red blood cells. On the other hand, Cd, which has various chronic toxic effects on the kidney as the final target organ, is mostly distributed first to the liver;[5–7] hence the liver is called the first target organ of Cd.[8] The behavior *in vivo* of Cd is known to change greatly when it is used in combination with SH compounds.[9] Therefore, Erg., which is also an SH compound in the living body, was expected to have some kind of preventive effect against the toxic effects caused by Cd. In the present work, we examined possible effects of Erg. on Cd-induced teratogenesis.

Experimental Methods

Animals——All animals were purchased from CLEA Japan, Inc. ICR-JCL virgin female mice (8 weeks of age) and SD-JCL virgin female rats (9 weeks of age) were used in experiments after prebreeding for a week. The mice were kept at a room temperature of 23±1°C and a humidity of 55—60% and were given chow prepared by CLEA Japan, Inc. (CA-1) and tap water *ad libitum*. Mating was carried out by placing the female animals at estrus in the cages of male rats (6 weeks of age) or mice (11 weeks of age) in the evening and letting them cohabit overnight. Female animals whose plug or vaginal smear contained spermatozoa on the next morning were used in the experiment as animals in day zero of pregnancy.

Samples and Methods of Administration——1) Effects of Maternal Administration of Erg. on Fertilization, Nidation and Teratogenesis in Rats: Erg. (Sigma) was administered *i.p.* at a dose of 16 or 8 mg/2 ml of saline/kg. The dosage after pregnancy was fixed at that based on the body weight on day zero of pregnancy. Saline solution was administered to the control group. The *i.p.* administration was conducted every day for 14 d before pregnancy and 8 d thereafter.

2) Effects of Paternal Administration of Erg. on Nidation and Teratogenesis in Rats: Erg. was administered *i.p.* every day at a dose of 8 or 0.8 mg/2 ml of saline/kg to male rats (6 weeks of age). These animals were mated with female rats (7 weeks of age) on day 67 of administration, sacrificed by means of a blow on the head on day 71, and autopsied. Fetuses from the female pregnant rats were examined for teratogenesis.

3) Examinations for Teratogenesis by Erg. in Female Mice and for Preventive Activity of Erg. against Teratogenesis caused by Cd: Erg. was administered *i.p.* every day at a dose of 80 mg/10 ml/kg during days 5 to 9 of pregnancy. Saline solution was administered to the control group. A single *i.p.* injection of Cd was given at a dose of 2.0 mg/ml/kg, concomitantly with the Erg. injection on day 7 of gestation.

Examination for Teratogenesis——Fetuses were examined as described in previous reports.[10,11] The mice and the rats were sacrificed by ether anesthesia on day 17 and 21 of pregnancy, respectively, and subjected to Cesarean section. The total number of nidations and the number of dead fetuses were counted. Living fetuses were examined macroscopically for possible external abnormalities after measurement of the body weight. Half of the living fetuses was fixed in 99% ethanol, stained with alizarin red S according to Dawson's method,[12] and examined for skeletal abnormalities under a dissection microscope. The other half was sliced serially according to Wilson's method[13] after fixation in Bouin's solution and examined for possible visceral abnormalities.

Distribution of ^{109}Cd——Maternal mice were administered ^{109}Cd (New England Nuclear Co., USA) *i.p.* at a dose of 50 μCi/2 mg Cd/kg on day 7 of pregnancy. The animals were sacrificed on day 8 or 17, and the liver, kidney, uterus, fetus, placenta, *etc.* were removed. The liver was excised after saline perfusion *in situ*. Each organ was washed with cold saline, freed of excess water by blotting with a filter paper, and weighed. For gel filtration studies, each organ removed on day 17 was homogenized with 3 volumes of 0.25 M sucrose in 50 mM Tris–HCl buffer (pH 8.0) and centrifuged at 105000 *g* for 60 min. The supernatant was charged on a Sephadex G-75 column. Radioactivity in each fraction or tissues prepared by dissolution in 10 N NaOH under heating was counted with the Aloka auto well scintillation counter.

Results

Effects of Erg. on fertilization, fetal mortality and teratogenesis were examined in male and female rats (Table I). No marked effects of Erg. on the number of nidations, the number of living fetuses, and the fetal body weight were observed after maternal or paternal injections.

Table I. Effect of Ergothioneine on Rat Fetuses after Maternal and Paternal Administration

Administration of ergothioneine (mg/kg/d)		No. of litters	No. of implants	No. of live (%)	Dead or resorbed (%)	Fetal[b] Body weight (g) Male	Female	No. of malformations/ No. of examined External	Skeletal	Internal
Maternal[a]	16	4	59	55(93)	4(6.8)	4.59±0.50	4.51±0.31	0/55	0/21	4/34[c]
	8	5	71	69(97)	2(2.9)	4.85±0.19	4.61±0.23	0/69	0/25	0/44
	0	5	75	67(89)	8(10.8)	4.90±0.55	4.71±0.48	0/67	0/24	2/43[c]
Paternal[b]	8	8	99	95(96)	4(4.0)	5.47±0.41	5.15±0.33	0/95	0/66	1/29[c]
	0.8	7	84	80(95)	4(4.7)	5.29±0.26	4.77±0.32	0/80	0/57	0/23
	0	8	109	105(96)	4(3.7)	5.25±0.44	4.95±0.41	0/105	0/70	2/35[c]

a) Daily *i.p.* injection of ergothioneine was done during 14 d before pregnancy and 8 d thereafter.
b) Male rats received daily *i.p.* injection of ergothioneine and were mated with female rats on day 67 of administration. Fetuses from the females were examined.
c) Hydronephrosis.
d) Mean±S.D.

A tendency for decreased fetal mortality, however, was observed after maternal injections. The incidences of external, skeletal, and visceral malformations were also the same in the Erg. group as in the control group; therefore Erg. appears not to cause teratogenesis. Visceral malformations observed in a few cases of the control group and the Erg. group were all hydronephrosis. No effects of Erg. were observed on the rate of increase in the maternal body weight, the mating rate, and the number of corpora lutea (not shown).

As stated above, Erg. administered to rats showed no effects on ovulation, nidation, living fetuses, and teratogenesis.

The animal species used was changed to mice, and the effects of maternal Erg. injection were examined; no effects of Erg. (80 mg/kg) were observed in mice, the number of nidations, living fetuses, dead fetuses, and the body weight of living fetuses being the same as in the control group and the incidence of teratogenesis being 0% as in the control group (Table II).

Thus, the effects of Erg. on teratogenesis caused by Cd were examined under identical conditions (Table II). Administration of Cd (2 mg/kg) caused death or fetal resorption in 79 of 257 cases. The fetal body weight decreased significantly, and as regrads teratogenesis, exencephalia and open eye occurred in as many as 64% and 31.5% of the cases, respectively.

Table II. Preventive Effects of Maternal Administration of Ergothioneine on Mouse Embryotoxicity of Cadmium Chloride

Treatment	No. of litters	Total implants	No. of live	Dead or resorbed (%)	Fetal body weight (g) (mean ± S.D.) Male	Female	Malformation (%) Exe.	O.E.
Saline	5	56	54	2/56 (3.6)	1.47 ±0.19	1.43 ±0.20		
Erg.[a]	4	45	44	1/45 (2.2)	1.49 ±0.22	1.44 ±0.21		
Cd[b]+saline	20	257	178	79/257[d] (30.7)	1.24[c] ±0.11	1.17[c] ±0.13	114/178[d] (64.0)	56/178[d] (31.5)
Cd[b]+Erg.[a]	19	220	175	45/220[e] (20.5)	1.36 ±0.18	1.22 ±0.22	78/175[f] (44.6)	29/175[f] (16.6)

Exe.; Exencephalia. O.E.; Open eye.
a) Ergothioneine was administered *i.p.* every day at a dose of 80 mg/kg during days 5 to 9 of pregnancy.
b) Single *i.p.* injection of Cd was done at a dose of 2.0 mg/kg on day 7 of pregnancy.
Significant differences against saline group (*c*); $p < 0.05$, *d*); $p < 0.005$).
Significant differences against Cd+Saline group (*e*); $p < 0.025$, *f*); $p < 0.005$).

Table III. Preventive Effect of Ergothioneine on Skeletal Abnormalities in Mouse Fetuses

Treatment	Cd[a]+Saline	Cd[a]+Erg.[b]
No. of Fetuses examined	76	69
Vertebra		
Cervical	35	10[c]
Thoracic	51	23[c]
Lumbar	23	11[d]
Rib		
Fusion	26	20
Division	6	1
Absence	6	1
Sternum		
Asymmetry	10	6
Skeleton malformed (%)	64	35[c]

Significant differences (*d*; $p < 0.05$, *c*; $p < 0.005$)
a,b) Under the conditions described in Table II.

Table IV. Distribution of Radioactivity in Mice 1 or 10 d after Maternal Administration of $^{109}Cd^{a)}$

		Days[b]	Liver	Kidney	Uterus	Fetus+placenta
Distribution (%)	Saline	1	38.8± 2.1	3.2± 0.2	0.6±0.05	Trace
		10	35.7± 1.9	4.5± 0.2	0.9±0.07	Trace
	Erg.[c]	1	38.6± 2.2	3.3± 0.2	0.7±0.04	Trace
		10	39.4± 1.6	4.6± 0.2	0.8±0.09	Trace
Specific activity (cpm/mg)	Saline	1	389.3±21.8	154.6± 7.5	59.9±3.2	47.0±7.9
		10	280.0±26.8	190.2±10.6	16.8±3.0	.....
	Erg.[c]	1	367.8± 9.8	154.2± 6.5	62.0±3.1	54.6±4.9
		10	290.8±31.4	180.9±15.6	16.1±2.5	.....

Values shown are means±S.D. (n=9).
a) Single *i.p.* injection of ^{109}Cd was done at a dose of 50 μCi/2 mg/kg on day 7 of pregnancy.
b) Days after maternal administration of ^{109}Cd.
c) Ergothioneine was administered *i.p.* every day at a dose of 80 mg/kg during days 5 to 9 of pregnancy.

Table V. Distribution of Radioactivity in Mouse Placenta 10 d after Maternal Administration of ^{109}Cd

Placenta	$^{109}Cd^{a)}$+Saline (cpm/mg)	$^{109}Cd^{a)}$+Erg.[b] (cpm/mg)
Normal	3.41±0.57	3.24±0.36
Exencephalia	3.52±0.26	3.45±0.22
Dead or resorbed	5.89±0.07[c]	5.93±0.68[c]

Values shown are means±S.D. (n=5 or more).
Significant differences from normal group (c; $p<0.001$)
a), *b*) Under the conditions described in Table IV.

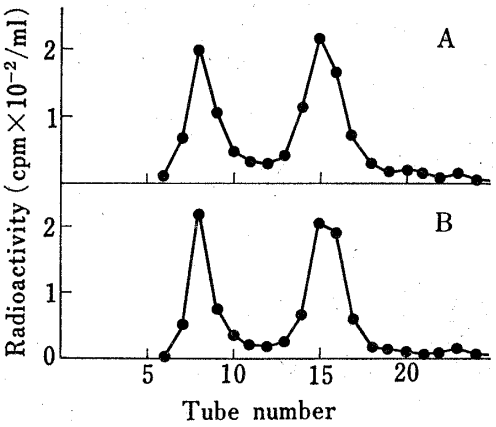

Fig. 1. Elution Profiles of 105000 *g* Supernatant of Placenta, 10 d after Maternal Administration of ^{109}Cd

(A): $^{109}Cd^{a)}$+saline, (B): $^{109}Cd^{a)}$+Erg.[b]
Placentae were homogenized and centrifuged at 105000*g* for 60 min. The supernatant was charged on a Sephadex G-75 column. Column size; 1.5×40 cm. Eluent; 50 mM Tris-HCl buffer (pH 8.0).
a),*b*) Under the conditions described in Table IV.

A combined administration of Cd and Erg. caused a significant decrease (20.5%, $p<0.025$) in the fetal mortality. Further, Erg. very significantly inhibited ($p<0.005$) Cd-induced injuries such as exencephalia and open eye. The combined administration was also found to prevent the significant decrease in the fetal body weight ($p<0.05$) caused by Cd, and body weight remained at the level in the control group. Table III lists the skeletal malformation in 76 cases of the Cd group and in 69 cases of the group given Cd plus Erg. In the former group, abnormalities were found in many cases in cervical, thoracic, and lumbar vertebrae, and abnormalities such as fusion, division and absence were observed in the rib. Asymmetry of the sternum was also observed. Abnormalities in thoracic vertebrae were observed in as many as 51 of 76 cases. A combined administration of Cd and Erg. exerted a marked preventive effect on abnormalities in the backbone and a very significant preventive effect on abnormalities in the cervical ($p<0.005$), thoracic ($p<0.005$), and lumbar vertebrae ($p<0.05$), as well as markedly reducing skeletal malformations caused by Cd.

Erg. proved significantly inhibit the ability of Cd to induce both external and skeletal malformations in mice. It was confirmed that Erg. itself has no teratogenic action in mice, in

the same way as for rats.

The mechanism of action of Erg. was thus studied with the use of ^{109}Cd in terms of the *in vivo* behavior of Cd. Table IV shows the results obtained by examining the distribution *in vivo* of ^{109}Cd 24 h and 10 d after its administration on day 7 of pregnancy. There were no differences between the Erg. group and the control group as regards the distribution of ^{109}Cd in the liver, kidney and uterus. The distribution and specific radioactivity of ^{109}Cd in fetuses on day 17 of pregnancy and 10 d after its administration were both small.

Table V summarizes the distribution *in vivo* of ^{109}Cd in the placenta 10 d after its administration. There were no differences between the placenta of normal fetuses and of fetuses with exencephalia as regards the distribution of ^{109}Cd. The distribution of ^{109}Cd in the placenta of dead fetuses, however, was increased very significantly. The distribution of ^{109}Cd, however, was not at all affected by Erg. administration in this case either, there being no differences between the Erg. group and the control group. As described above, under these conditions, Erg. not only had no effect on the distribution of Cd in maternal internal organs but also had little effect on the elimination of Cd in feces and urine, (not shown); therefore, the inhibitory action of Erg. on Cd-induced teratogenesis is considered not to be due to changes in the *in vivo* distribution of Cd induced by Erg. Thus, we next compared the elution profiles of column chromatography on Sephadex G-75 in order to examine whether there is any difference in the form of Cd. In the supernatant fractions of the liver and kidney, as is already well known,[14] almost all Cd exists in a form bound to metallothionein fraction (MT-fr), there being no difference between the two groups. Fig. 1 also shows the results for the placenta. In the placenta, the distribution to a high molecular fraction (HM-fr) eluted in the void volume of the column was greater than those to the liver and kidney; Erg. administration did not influence the patterns of distribution of ^{109}Cd to a HM-fr and MT-fr.

Discussion

The possible effects of Erg. on Cd-induced teratogenesis were examined in this work. It was confirmed that Erg. itself has no effects on ovulation, nidation, living fetuses, or teratogenesis. However, the combined administration of Erg. not only reduces the fetal mortality due to Cd but also significantly reduces the incidence of external abnormalities, such as exencephalia and open eye, and skeletal malformations as represented by dorsal vertebra abnormalities.

The *in vivo* behavior of Cd is known to depend on the presence of –SH compounds; for example, combined administration of Cd and cysteine or glutathione increases the distribution of Cd to the kidney,[9] and the presence of cysteine increases the transfer of Cd in the small intestine.[15] Therefore, assuming that the effect of Erg. is due to a decrease in the transfer of Cd to the placenta or fetuses, we examined the *in vivo* distribution of ^{109}Cd. However, no changes were observed in the maternal distribution of ^{109}Cd or in the distribution of Cd to the placenta and fetuses in the Erg. plus Cd group compared to the Cd group.

Although onset of Cd toxicity is considered to be influenced by whether it exists in a bound form (MT form), or in a free form,[16,17] there was no difference between the forms of Cd in the Erg. group and the control group, either in the liver or in the placenta. Therefore, the inhibitory effect of Erg. on the teratogenesis caused by Cd is apparently not due to changes in the amount of Cd transferred to the placenta and fetuses or to changes in the MT concentration in the placenta.

Zinc, which is an essential metal, is also known to markedly inhibit the teratogenesis caused by Cd.[18] This metal is considered not to influence the transfer of Cd from the maternal to fetal bodies but rather to reduce the Cd toxicity at embryonic sites; alternatively, the transfer of Zn necessary for the fetal growth may be blocked even if there are no changes in the amount of Cd transferred, since the malformation occurs even in a state of Zn-deficiency.[19] The mechanism of the inhibitory action of Erg. remains to be clarified, but the same

possibility can be considered in the case of this compound. The possibilities are as follows: Erg. facilitates the passage of (an) essential metal (s) through the placenta; Erg. inhibits the onset of abnormalities due to Cd at the site of their occurrence; Erg. exerts inhibitory effects on possible secondary injuries to fetuses caused by Cd, which is known to induce various toxic biochemical changes in the liver.[20] These possibilities, as well as the possibility of direct interaction between Erg. and Cd, remain to be investigated.

References and Notes

1) Part VII: H. Kawano, F. Higuchi, T. Mayumi, and T. Hama, *Chem. Pharm. Bull.*, **30**, in press (1982).
2) T. Mayumi, H. Kawano, Y. Sakamoto, E. Suehisa, Y. Kawai, and T. Hama, *Chem. Pharm. Bull.*, **26**, 3772 (1978).
3) H. Kawano, M. Otani, K. Takeyama, Y. Kawai, T. Mayumi, and T. Hama, *Chem. Pharm. Bull.*, **30**, 1760 (1982).
4) H. Kawano, F. Higuchi, T. Mayumi, and T. Hama, *Chem. Pharm. Bull.*, **30**, in press (1982).
5) K. Tanaka, K. Sueda, S. Onosaka, and K. Okahara, *Toxicol. Appl. Pharmacol.*, **33**, 258 (1975).
6) D. Kello and K. Kostial, *Environ. Res.*, **14**, 92 (1977).
7) M. Cempel and M. Webb, *Biochem. Pharmacol.*, **25**, 2067 (1976).
8) Y. Sayato, A. Hasegawa, and M. Ando, *Eiseikagaku*, **17**, 398 (1971).
9) N. Yasuda, T. Fujita, and S. Morimoto, *Yakugaku Zasshi*, **94**, 153 (1974).
10) K. Okamoto, Y. Kobayashi, K. Yoshida, Y. Nozaki, Y. Kawai, H. Kawano, T. Mayumi, and T. Hama, *Cong. Anom.*, **18**, 227 (1978).
11) K. Okamoto, K. Hoshino, A. Yoshida, Y. Kawai, T. Mayumi, and T. Hama, *Cong. Anom.*, **20**, 359 (1980).
12) A.B. Dawson, *Stain Technol.*, **1**, 123 (1926).
13) J.G. Wilson and J. Warkany, "Teratology: Principles and Techniques," The University of Chicago Press, Chicago, 1965, pp. 251—277.
14) K. Tanaka, K. Sueda, and K. Okahara, *Eiseikagaku*, **20**, 98 (1974).
15) S. Kojima, M. Kiyozumi, and M. Kamiya, *J. Food Hyg. Soc.*, **19**, 553 (1978).
16) M.G. Cherian, *Toxicology*, **17**, 225 (1980).
17) M.G. Cherian, and R.A. Gayer, *Life Sciences*, **23**, 1 (1978).
18) V.H. Ferm, O.W. Haulsen, and J. Urban, *J. Embryol. Exp. Morph.*, **22**, 107 (1969).
19) G.P. Samarawickrama, "The Chemistry, Biochemistry and Biology of Cadmium," 1st ed., ed. by M. Webb, Elsevier/North Holland, Amsterdam, 1979, pp. 381—392.
20) G.P. Samarawickrama, "The Chemistry, Biochemistry and Biology of Cadmium," 1st ed., ed by M. Webb, Elsevier/North Holland, Amsterdam, 1979, pp. 370—373.