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Toxicity of Copper Salts in Hamster Embryonic Development

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The intravenous injection of copper salts into pregnant hamsters on the eighth day of gestation caused an increase in embryonic resorptions as well as the appearance of developmental malformations in surviving offspring. Malformations of the heart appeared to be a specific result of the toxicity of these copper compounds. Copper in a chelated form (copper citrate) was only slightly more embryocidal, but considerably more embryopathic than that in the uncomplexed form (copper sulfate). Additional studies on the permeability of the early hamster placenta during the critical stages of organogenesis (Day 8) revealed that the placenta was permeable to radioactive copper (citrate form), indicating that this metal may have a direct teratogenic effect upon the developing embryo.

The reaction of the mammalian embryo to copper represents a biological enigma. On the one hand, there is evidence that a deficiency of copper during gestation is detrimental to embryonic survival in rat and chick embryos (O'Dell *et al.*, 1961; Dutt and Mills, 1960). Rat embryos from mothers maintained on copper-deficient diets show consistent patterns of edema and subcutaneous hemorrhage, as well as some skeletal abnormalities and abdominal hernias. Chick embryos from hens maintained on diets low in copper die early in incubation and show evidence of inhibited development, mesenchymal abnormalities, and hemorrhages similar to those of the rat embryo (O'Dell, 1968). On the other hand, the presence of small amounts of excess copper in the form of intrauterine loops or wires composed primarily of metallic copper has a detrimental effect on mammalian development, preventing implantation and blastocyst development (Chang *et al.*, 1970, Zipper *et al.*, 1969). In an attempt to define more precisely the role of copper in mammalian development, and especially its potential teratogenic and/or embryopathic effect on the mammalian embryo, we have studied the effects of excess copper during the critical stages of embryogenesis in the golden hamster. This report also includes studies on the permeability of the rodent placenta to radioactive copper during these early critical stages of embryogenesis.

MATERIALS AND METHODS

Pregnant golden hamsters (*Cricetus auratus*) were purchased from the Lakeview Hamster Colony. Under our scheme of pregnancy timing, the day following the evening of mating is considered to be the first day of gestation. All animals were injected intravenously using the lingual vein, while under pentobarbital anesthesia, on the morning of the eighth day of gestation. The stock solutions of copper salts used were 0.125 M $CuSO_4$ (0.80 mg Cu^{+2}/ml) and 0.04 M $Cu(citrate)_2$ (0.25 mg Cu^{+2}/ml). The $Cu(citrate)_2$ complex was formed by adding 1 vol of 0.1 M $CuCl_2$ to 2 vol of citric acid. The pH was adjusted to 7.0 with 2 N NaOH and the solution diluted with distilled water. All chemicals were analytical reagent grade. The injected volumes of control, $CuSO_4$ and $Cu(citrate)_2$ solutions were never greater than 1.0 ml/100 g body wt, although the total amounts of metal ion injected were varied as shown in Table 1. Dilution of stock Cu^{+2} solutions was made with demineralized water. The animals were sacrificed 4 or 5 days after injection. Each gestation

TABLE 1

EFFECT OF COPPER SALTS ON HAMSTER EMBRYONIC DEVELOPMENT

Dose level mg Cu/kg	No. of mothers treated	No. of gestation sacs	No. of living embryos (%)	No. of resorptions (%)	No. of abnormal embryos (%)
Copper sulfate					
2.13	16	210	155 (74)	55 (26)	12[a] (6)
4.25	3	49	7 (14)	42 (86)	4[b] (8)
7.50	3	30	0 (0)	22 (74)	—
10.0	2	Maternicidal			
Copper citrate					
0.25–1.50	13	172	143 (83)	29 (16)	4[c] (2)
1.80	6	81	48 (59)	33 (41)	14[d] (17)
2.20	8	99	65 (66)	34 (34)	35[e] (35)
4.0	2	Maternicidal			
Controls (demineralized water)					
0.5–1.0 ml/100 g	10	125	115 (92)	10 (8)	0 (0)

[a] 5 Thoracic wall hernias, 4 encephalocoeles, 2 spina bifida, 1 microphthalmia.
[b] 1 Exencephaly, 1 hydrocephalus, 1 abdominal hernia, 1 abnormal spinal curvature.
[c] 2 Tail abnormalities, 1 microphthalmia, 1 craniorrhachischisis.
[d] 13 Tail defects, 1 meningocoele.
[e] 25 Tail defects, 6 thoracic wall defects, 2 microphthalmia, 1 abdominal wall defect, 1 facial cleft.

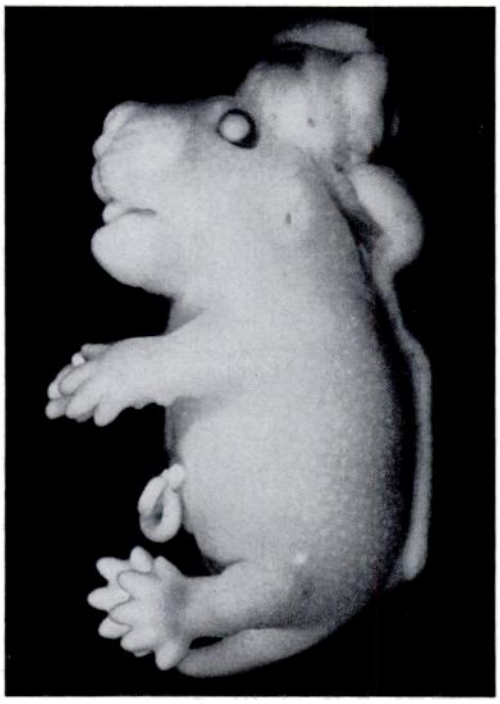

FIG. 1. 13-Day-old hamster fetus showing complete nonclosure of spinal cord and brain (craniorrhachischisis). Mother received 331 μg/100 g body wt of copper in the form of copper citrate on the eighth day of gestation. ×4.

sac was carefully examined for evidence of embryonic resorption and all living embryos recovered. Embryos were examined under a binocular dissecting microscope and all morphological abnormalities were recorded.

A second series of experiments were performed to determine the distribution of injected radioactive copper in pregnant hamsters during the critical stages of embryogenesis. Six pregnant animals were injected on the eighth day of gestation as described above. Copper (0.25 mg Cu^{+2}/100 g) was introduced in the form of the $Cu(citrate)_2$ complex to which was added 6 μCi of $^{64}Cu^{+2}$/ml. The radioisotope was obtained from New England Nuclear Corp. as $^{64}Cu(NO_3)_2$ in 1 N HNO_3 initially calibrated to contain 34.4 mCi/mg copper.

These animals were sacrificed on Day 9, 24 h after injection. Tissue samples were obtained and radioassayed as previously described (Ferm *et al.*, 1969). Sample counts were compared with those of a standard $^{64}Cu^{+2}$ solution measured concurrently to compensate for decreases in radioactivity resulting from the short half-life (12.8 h) of the isotope.

RESULTS

The embryocidal and teratogenic effects of $CuSO_4$ and $Cu(citrate)_2$ at various dosage levels are listed in Table 1. Frequencies of malformations appear as footnotes. Both

forms of Cu^{+2} are teratogenic, but the citrate complex is more teratogenic than the free metal ion. Teratogenic levels of Cu^{+2} introduced in either form result in increased fetal resorption rates. Maternicidal doses of $CuSO_4$ and $Cu(citrate)_2$ are also shown in Table 1. Again, the complexed species of Cu^{+2} is more potent.

The spectra of embryonic malformations are similar for both forms of Cu^{+2} with the notable exception of kinking of the tail extremity which is seen primarily with the citrate complex. Peculiar also to copper ion-treated hamsters is the relatively high incidence of thoracic and supraumbilical ventral hernias (Fig. 1). In addition, a few other malformations were found, including craniorrhachischisis (Fig. 3), microphthalmia, and facial cleft. Histological examination of the thoracic anomalies revealed that the heart is herniated through the opening in the thoracic wall (ectopia cordis).

The distribution of ^{64}Cu in maternal and embryonic tissues 24 h after intravenous injection is shown in Table 2. Results are given in terms of micrograms of $^{64}Cu/g$ of tissue. A sufficient number of counts was accumulated for each sample to keep the probable error in counting to less than

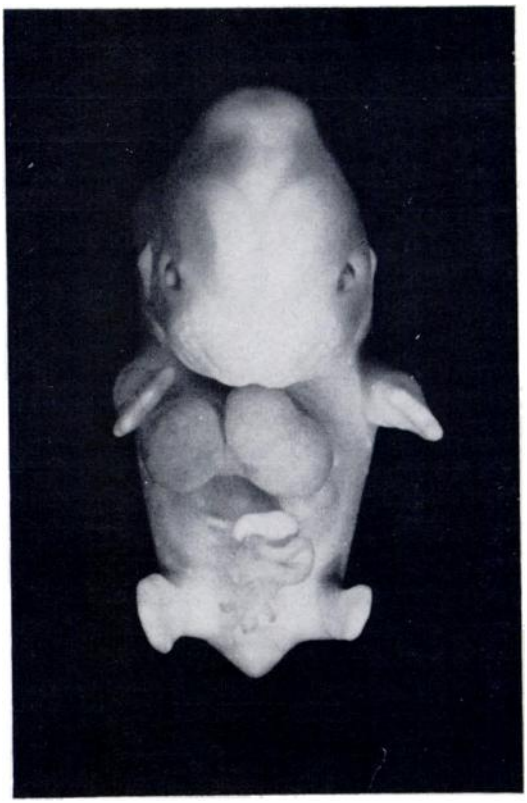

FIG. 2. 12-Day-old hamster fetus showing severe ectopia cordis. Mother received 115 μg/100 g body wt of copper in the form of copper citrate on the eighth day of gestation. Note shortened and kinked tail. ×3.

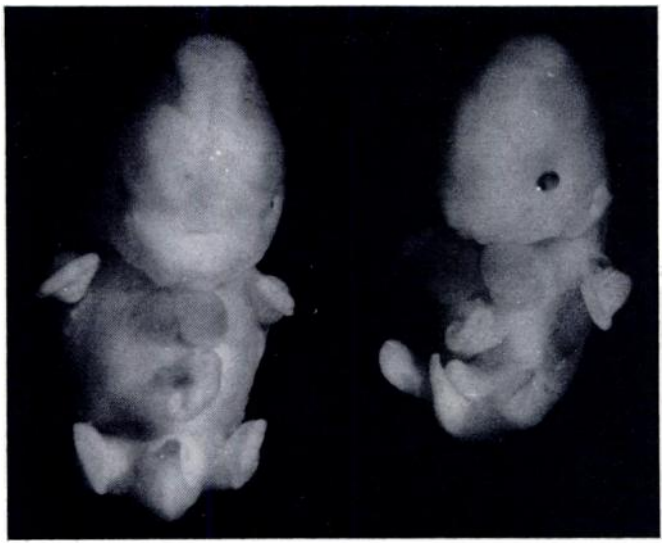

FIG. 3. 12-Day-old hamster fetus littermates both showing ectopia cordis. Mother received 168 μg/100 g body wt of copper in the form of copper sulfate on the eighth day of gestation. An additional littermate had an identical malformation. ×3.

TABLE 2

THE DISTRIBUTION OF ^{64}Cu IN THE PREGNANT HAMSTER 24 H AFTER INTRAVENOUS INJECTION OF $^{64}Cu(Citrate)_2$ ON THE EIGHTH DAY OF GESTATION

	Micrograms of ^{64}Cu g/tissue				
Hamster number	Maternal blood	Maternal liver	Uterus	Placenta	Embryo
6406	0.62	14.0	0.53	1.59	0.69
6407	0.54	12.3	0.45	1.67	0.61
6408	0.57	13.3	0.70	1.40	0.59
6409	0.59	14.1	0.63	1.33	0.80
6410	0.52	12.5	0.55	1.39	1.17
6411	0.48	10.6	0.31	1.44	1.00
Mean	0.55	12.8	0.53	1.47	0.81
Standard error	±0.02	±0.53	±0.06	±0.05	±0.09

±1%. However, in the specific case of the embryo samples, the ratio of excess counts to background counts was such that the error could be as great as ±20% of the indicated value.

DISCUSSION

Copper is an essential component of a number of metalloenzyme systems involved in catalyzing oxidative metabolic reactions. Because of this it may well play an important part in certain stages of embryonic organogenesis. Cupric ion in excess is also well known as a toxic agent, exerting its effects at the metabolic level by inhibiting enzyme and possibly by damaging subcellular membrane structure (Chvapil *et al.*, 1972). For these reasons its teratogenic action in hamsters is not entirely unexpected. Our observations of the serious malformations of the fetal heart (ectopia cordis) fit well with those reports of site-specific anomalies induced by other heavy metals (Ferm, 1972). However, the occurrence of ectopia cordis appears to be unique for Cu^{+2} among many other metals tested in this animal system.

In contrast to our data which show that Cu^{+2} introduced intravenously on Day 8 of pregnancy causes embryonic abnormalities, copper wire placed in the hamster uterus after implantation of the blastocysts is nonteratogenic (Chang and Tatum, 1973). Because cupric ions are released from metallic copper *in utero* (Okereke *et al.*, 1972), the effects of Cu^{+2} on hamster embryos might be determined at least in part by the route of entry. The teratogenicity of Cu^{+2} injected as the citrate chelate far exceeds that of the metal salt (see Table 1). This most likely results from a difference in the mode of distribution of copper ion injected in the chelated as compared to the uncomplexed state. Uncomplexed copper ion injected into the blood stream is immediately bound to serum albumin and carried to the liver. It is later released, bound tightly to ceruloplasmin (Bearn and Kunkel, 1955). On the other hand, copper in the form of a chelate must be carried more directly to maternal body tissues as well as to the placenta and the developing embryos. In support of this statement are the findings of Walshe and co-workers in their study of the effects of penicillamine on copper distribution (Walshe, 1966). Also, Koutensky *et al.* (1971) have shown that diethyldithiocarbamate (DDC) significantly increases the toxicity of copper in mice, and changes its pattern of tissue distribution.

On the ninth day of gestation, 24 h after injection of $^{64}Cu^{+2}$ as the citrate complex all tissues examined show detectable amounts of $^{64}Cu^{+2}$. Maternal liver, placenta, and embryos had concentrations of $^{64}Cu^{+2}$

which are 23.1, 2.7, and 1.5 times that of whole blood, while levels in the uterus were not significantly different from blood levels. Although the error in counting embryo samples may have been ±20%, our data definitely show that the placenta is permeable to $^{64}Cu^{+2}$ during the critical period when other metal ions produce a teratogenic effect (Ferm, 1972). This is consistent with the notion that Cu^{+2} teratogenicity is due to a direct effect of the metal ion on specific embryonic sites.

The amount of $^{64}Cu^{+2}$ present in the embryonic tissue 24 h after injection averaged less than 1.0 μg/mg (see Table 2), which represents an increase of approximately 40–70% over the endogenous level of copper, providing hamster levels of the metal ion are the same as those noted for adult vertebrates (Adelstein and Vallee, 1962). If this amount of copper is representative of the level in the embryonic tissues in the 24-h period during which abnormalities occur, then the sites of attack by the metal ion must be relatively few in number and very sensitive to copper.

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RECOMMENDED REVIEWS

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