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Embryotoxicity of silver ions is diminished by ceruloplasmin—further evidence for its role in the transport of copper

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The effect of alimentary administration of silver salts upon embryogenesis in rats was studied. Feeding of female rats throughout the term on a regular diet supplemented with AgCl did not cause alterations of their physiological functions, despite the fact that enzymatically active copper-containing ceruloplasmin (CP) was eliminated from the blood plasma. However, developmental abnormalities of embryos, their prenatal death or the 100% mortality of the newborns in the first 24 h of life was seen. Copper content in placenta and fetal tissues was strongly diminished. Cu, Zn-superoxide dismutase (SOD) activity decreased in cytoplasm of embryonic cells along with a drop, though less pronounced, in the tissues of the pregnant females. Embryotoxicity of AgCl was seriously diminished by repetitive injections of native CP to the pregnant rats. Such treatment resulted in an increase of SOD activity in placenta and embryonic tissues. The mortality of the newborns also became less. It is suggested that the embryotoxic effect of AgCl is caused by its ability to interfere with copper metabolism, in particular by altering the copper-transporting function of CP.

Keywords: ceruloplasmin, copper metabolism, embryogenesis, metal toxicity

Introduction

The mechanisms of absorption, transport, distribution and detoxication of heavy metal ions, referred to as essential elements, have been so far insufficiently studied.

Among these essential elements, copper plays a role of great importance, since its ions are incorporated into the active centers of such enzymes as cytochrome *c* oxidase, superoxide dismutase, lysyloxidase, tyrosinase, ceruloplasmin (CP), etc. Alterations in copper metabolism underlie such hereditary maladies as Menkes's syndrome and hepatolenticular degeneration or Wilson's disease (WD) (Danks 1977, 1980, Owen 1981). Both copper deficiency and its excess are unfavorable conditions for an organism (Hurley & Keen 1979, Danks 1980, McBrien 1980, Owen 1981). However, not much is known about the particular mechanisms underlying each of these conditions, as no proteins definitely participating in the regulation of copper storage and transport have been identified. It has been acknowledged since long ago that CP (ferro-O_2-oxidoreductase; EC 1.16.3.1.) is the main copper-transporting protein of the vertebrate blood plasma (Frieden 1979). Indirect evidence in favor of the copper-transporting function for this protein has been obtained (Marceau & Aspin 1973a, b, Hsieh & Frieden 1975). However, so far it has not been directly demonstrated that it is CP that transports copper from liver, where this protein is synthesized, to other tissues. In some way this function of CP was confirmed by discovering specific CP-binding receptors on cellular membranes (Barnes & Frieden 1984, Kataoka & Tavassoli 1984, Puchkova *et al.* 1990).

In our studies of the effect of silver ions on the properties of CP in animals, we demonstrated that 2–3 weeks of feeding the rats on regular diet supplemented with silver salts results in virtually complete elimination from the blood plasma of the copper-containing CP able to perform as an oxidase (Pribyl *et al.* 1982, 1989). In the meantime, the content of immunoreactive CP is much less decreased. Electrophoretic properties of CP in such

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animals differed from those of normal rat protein—its copper ions were substituted for silver and one of its molecular forms was structurally altered. These changes, when accumulated, result in a sharp decrease of copper content in plasma (normally about 98% of all plasma copper is provided by CP). In some way this state is similar to the alimentary deficiency of copper; however, despite the notable alterations in the properties of CP and the low plasma level of copper, the silver-treated animals did not differ from the controls with respect to their body mass, behavior, ability to take food, etc.

Considering all that was said above, it was interesting to study the effect of silver salts and of the altered properties of CP upon embryogenesis, which requires an enhanced transport of copper towards the growing tissues. Pregnant rats fed on a silver-containing diet were used as a model for our study.

Materials and methods

Inbred albino female rats (body mass 180–200 g) were kept on a regular diet with a balanced content of microelements. To achieve the toxic effect, 50 mg of AgCl per animal per day was mixed in the food given to the group under trial. Water was given *ad libitum*.

The day when spermatozoa were found in the vaginal smears was counted as the first day of the term.

A sterilized commercial preparation of 95% pure human CP produced by the Pasteur Institute of Epidemiology and Microbiology (Saint Petersburg) according to the technology elaborated in the Institute for Experimental Medicine was injected intraperitoneally every 48 h in portions of 0.3 ml at a concentration of 35 mg/ml.

On the 20th day of term, animals were sacrificed by dislocation of cervical vertebrae, the uterus was dissected, the number of live and dead fetuses as well as those with malformations was estimated, and the *corpora lutei* were counted in the ovaries. The fetuses were weighed and the condition of their organs was evaluated using the modified method of Wilson (1965).

Copper content in tissue samples was measured using atomic absorption spectrometry (AAS-1; Karl Zeiss, Jena, Germany) after drying the tissues in vacuum upon phosphoric anhydride. Samples of known mass were burned in a mixture of $HClO_4$:H_2SO_4:H_2O (100:25:125, v/v) and then dissolved in deionized water to always achieve the same volume.

Cytochrome *c* oxidase (COX) activity was measured in the mitochondrial cellular fraction (Smith 1955). Mitochondria were isolated as described by Merle & Kadenbach (1980).

The activity of Cu, Zn-superoxide dismutase (Cu, Zn-SOD) was measured in the soluble cellular fraction as described by Vassiliev *et al.* (1988).

Oxidase activity of rat CP was tested in the samples of the blood serum according to the modified method of Ravin (Zakharova *et al.* 1983).

Concentrations of human CP in rat serum were measured by rocket immunoelectrophoresis 24 and 48 h after intraperitoneal injections of the protein (Laurell 1967).

Results

As we have shown previously (Pribyl *et al.* 1982, 1989), 1 week of feeding rats on minced and moistured standard food to which AgCl was added in amounts providing 50 mg of salt per animal per day resulted in virtually complete absence of the oxidase activity in their serum. The activity of CP in the serum of the pregnant females of the control group corresponded to its concentration of about 50 mg %.

The absence of the oxidase activity does not mean that CP had disappeared from blood circulation, since two immunoreactive components are revealed in the serum of the silver salt-treated rats by cross-immunoelectrophoresis with the antibodies against rat CP (Pribyl *et al.* 1989).

Table 1 shows the influence of the above-mentioned diet when it covered the time from day 7 to 15 of term (the period of organogenesis) upon the development of embryos. As can be seen, AgCl intake at that time did not affect the development of embryos. The indices of pre- and post-implantational death of embryos did not differ from those in controls. No external abnormalities were revealed among the live embryos at the moment of section (day 20 of term). The average body mass of fetuses corresponded to that of controls. The absence of visible embryotoxicity at this stage of embryogenesis is likely to be explained by the brevity of intervention (9 days), the duration of which correlates with the gradual decrease of the active CP content in the blood.

The results were different in the group of animals fed on the AgCl-supplemented diet throughout the whole term (day 1–20). As shown in Table 1, the pre-implantational embryolethality was the same as in the control group. The post-implantational lethality, however, was 36% ($P < 0.001$). Five of 145 embryos (2.5%) had visible abnormalities of their development, identified as omphalocele, eventeration and shortened tail. Body mass of embryos was substantially lower, the average at the moment of section being 1.75 g (the respective figure for the control group is 2.26 g; $P < 0.001$). The number of embryos having visceral damages was considerably higher in the trial group.

Since the embryotoxic effect of AgCl was obvious, the influence of this salt upon the survival of the newborn animals was studied. The results, presented in Table 2, clearly demonstrate that the newborn animals were unable to live, all dying within the first 24 h after birth.

The subsequent experiments were aimed at the elucidation of the mechanism of the embryotoxic effect of AgCl. Considering that silver salts cause a sharp decrease of the copper concentration in serum due to the alteration of CP biosynthesis, we first studied the copper content in various tissues of pregnant rats and embryos at the moment of section. The results are shown in Table 3.

Concentration of copper in the tissues of pregnant females getting AgCl with food throughout the entire term

Table 1. Embryotoxicity of AgCl

	Periods of intoxication		Control group	Historical control
	day 7–15	day 1–20		
No. of pregnant rats in the experiment	5	20	36	237
No. of *corpora lutei*	63	231	430	2758
No. of:				
implanted embryos	59	222	384	2537
pre-implantational deaths (also %)	4 (6.3)	9 (3.9)	46 (10.7)	221 (8.0)
post-implantational deaths (also %)	3 (5.0)	80 (36.0)	37 (9.6)	220 (8.7)
live fetuses	56	42	347	2317
visible abnormalities	0	5	0	0
Average mass of a fetus (g)	2.2	1.75	2.24	2.26
Visceral pathology				
No. of fetuses examined	51	98	150	659
of those having (also %):				
atrial dilatation	2 (3.9)	1 (1.0)	0	0
thoracal hemorrhage	0	8 (8.2)	1 (0.7)	78 (11.8)
abdominal hemorrhage	0	19 (19.4)	7 (4.7)	48 (7.3)
hepatic hemorrhage	0	8 (8.2)	0	6 (0.9)
hydronephrosis	2 (3.9)	30 (30.6)	8 (5.3)	8 (1.2)
cryptorchism	0	34 (34.7)	2 (1.3)	5 (0.8)

Table 2. The toxic effect of AgCl upon the postnatal development of animals and its correction with CP

	Effect of			Controls
	AgCl (day 1–20)	AgCl + CP (day 2–14)	AgCl + CP (day 8–21)	
No. of fertile rats	5[a]	6	6	4
No. of newborn animals	33	61	51	32
Of those, no. that died (also %)	33 (100)	21 (34.5)	3 (5.9)	0
Index of viability[b]	0	0.26	0.53	0.84
Index of lactation[c]	0	0.58	0.89	0.85
Average body mass on day 18 of life (g)	0	24.0	28.8	27.0
Index of the body mass increase[d]	0	3.9	3.6	3.2

Table 3. Copper content (mg g^{-1} of the dry weight of tissue) in the organs of animals upon introduction of AgCl separately and in combination with injections of CP

	Introduction throughout the whole term of		Controls
	AgCl	AgCl + CP	
Liver of adult female rats	11.5 ± 2.7	16.4 ± 1.7	17.5 ± 2.7
Heart of adult female rats	11.2 ± 1.3	16.3 ± 0.7	17.6 ± 2.6
Kidneys of adult female rats	8.6 ± 0.9	10.6 ± 0.7	13.9 ± 0.7
Placenta	0	4.3 ± 0.6	7.3 ± 0.6
Embryos	0	4.6 ± 0.6	5.8 ± 1.1
Blood serum of adult female rats (mg ml^{-1})	0	0.7 ± 0.05	1.3 ± 0.05

did not decrease considerably by the end of term. In the meantime, it became much lower in their serum, which correlated with the disappearance of the enzymatically active CP from the bloodstream. Copper content in placenta and embryonic tissues dropped to almost zero. It was interesting to observe the decrease of SOD activity in the tissues of the females and, particularly, in the embryonic tissues (see Figure 1). At the same time COX activity in the mitochondria of the adult rat liver and of the embryonic tissues was not changed (data not shown).

Although it seemed likely that the embryotoxic effect of AgCl is realized via the repression of the copper-transporting function of CP and, consequently, with copper deficiency in the developing tissues of embryos, a direct influence of silver ions, e.g. upon the placental tissues, could not be excluded. Therefore, in the next study we analyzed the effect of the active copper-containing CP introduced along with AgCl.

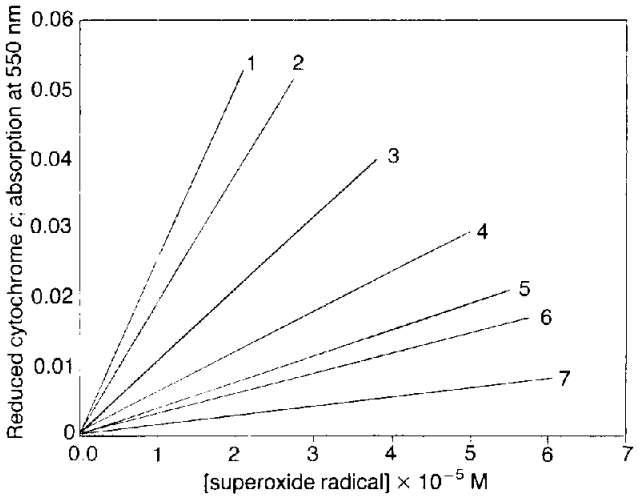

Figure 1. Kinetics of cytochrome *c* reduction upon injection into solution of O_2^- as described (Vassiliev *et al.* 1988). **1**, the reduction of cytochrome *c* without its protection by an O_2^- scavenger. Others are, respectively, the plots obtained upon adding into the reaction mixture of: **2**, an aliquot of cytoplasmic content from the tissues of embryos of AgCl-treated rats; **3**, similar sample, but from the rats also getting CP; **4**, a cytoplasmic aliquot from normal embryos; **5**, a cytoplasmic aliquot of AgCl-treated rat liver; **6**, similar to the previous sample, but from normal rats (control group); **7**, CuZn-SOD (2×10^{-9} M) isolated and purified from human erythrocytes.

Table 4. Correction of the embryotoxic effect of AgCl with injections of CP[a]

	Introduction throughout the whole term of	
	AgCl	AgCl + CP
No. of pregnant rats in the experiment	20	22
No. of *corpora lutei*	231	267
No. of:		
implanted embryos	222	262
pre-implantation deaths (also %)	9 (3.9)	5 (1.9)
post-implantation deaths (also %)	80 (36)	25 (9.5)
live fetuses	142	237
visible abnormalities	5	0
Average mass of a fetus (g)	1.75	2.04
Visceral pathology		
No. of fetuses examined	98	220
Of those having (also %):		
atrial dilatation	1 (1)	22 (10)
thoracal hemorrhage	8 (8.2)	24 (10.9)
abdominal hemorrhage	19 (19.4)	38 (17.2)
hepatic hemorrhage	8 (8.2)	23 (10.5)
hydronephrosis	30 (30.6)	8 (3.6)
cryptorchism	34 (34.7)	0

[a]The data concerning the control group are presented in Table 1.

Purified human CP was injected into the female rats at different stages of their pregnancy. As was evidenced by the results of rocket immunoelectrophoresis, the chosen regularity of injections provided that CP with oxidase activity circulated in the blood of rats under trial during the entire experiment, its concentration slightly decreasing by the end of the 48 h interval between injections.

Table 4 presents the results showing that 30 mg of CP per animal injected between day 2 and 14 of term caused partial normalization of the embryonic development. Embryo lethality did not differ from that in the controls, no abnormalities of fetal development were observed and the average body mass of fetuses was 2.04 g in contrast to 1.75 g of the 20 day fetuses in the rats getting AgCl without CP injections ($P < 0.001$). The share of fetuses with hydronephrosis and cryptorchism became less.

The postnatal survival was evaluated in the progeny of the rats treated with CP. The mortality of the newborns was 34% in contrast to 100% in the group without CP treatment. While the litter of the rats getting AgCl without CP died within the first 24 h after birth, those born from the CP-treated females survived for at least a few days. The vitality index for them was 0.26 and the lactation index was 0.58.

The effect of CP seems to be differently pronounced at different stages of pregnancy. Injections of CP between day 2 and 14 were less efficient than those between day 8 and 21. In the latter case, the postnatal mortality was only 5.9%. Besides, at day 18 of postnatal life the body mass of the surviving animals was higher than that in the control group and in the litter of the females treated with CP between day 2 and 14 (see Table 2).

Injections of CP affected the copper content in the tissues of the pregnant rats and their embryos. Table 3 shows that, while the increase of the copper amount in the tissues of adult animals was not so great, it was far more considerable in placenta and embryonic tissues. COX activity did not change, while that of SOD somewhat increased in the tissues of adults and, particularly, in those of embryos (see Figure 1).

Thus the evidence that CP-bound copper introduced into the organism affects embryogenesis was obtained, after which the adverse effects were studied, i.e. the excessive excretion of copper and iron from the organism by specific chelators, namely penicillamin and bipyridyl, as the animals were given AgCl. Bipyridyl was chosen, since, along with transport of copper, the main physiological role of CP is considered to be its ferroxidase function, i.e. oxidation of Fe^{2+} to Fe^{3+} and mobilization of iron from the tissue depots (Prasad 1978, Frieden 1979, Neifakh *et al.* 1988). It seemed likely that suppression of the ferroxidase function of CP upon introduction into the organism of excess amounts of silver should also alter the iron metabolism and, hence, provide even more grave consequences.

Table 5 illustrates the combined embryotoxic effect of

Table 5. Embryotoxic effect of bipyridyl and penicillamin introduced throughout the whole term along with AgCl

	AgCl	Bipyridyl	AgCl + Bipyridyl	Penicillamin	AgCl + penicillamin	Controls
No. of:						
animals under trial	20	14	15	7	9	36
implanted embryos	222	159	65	94	94	384
postimplantational deaths (also %)	80 (36)	11 (6.9)	86 (55)	2 (3.1)	74 (78.7)	37 (9.6)
live embryos	142	148	70	63	20	347
visible abnormalities (also %)	5 (3.5)	24 (16)	18 (25.7)	0	1 (5)	0
Average mass of a fetus (g)	1.75	1.90	1.70	2.23	1.76	2.24
Visceral pathology						
The number of fetuses examined	98	72	70	68	20	150
Of those with (also %):						
atrial dilatation	1 (1)	3 (4.2)	0	3 (4.4)	0	0
thoracal hemorrhage	8 (8.2)	0	18 (25.7)	1 (1.5)	0	1 (0.7)
abdominal hemorrhage	19 (19.4)	9 (12.5)	17 (24.3)	12 (17.6)	2 (10)	7 (46.7)
hepatic hemorrhage	8 (8.2)	1 (1.4)	5 (7.1)	0	0	0
hydronephrosis	30 (30.6)	7 (9.7)	21 (30)	1 (1.5)	14 (70)	8 (5.3)
cryptorchism	34 (34.7)	0	13 (18.6)	0	9 (45)	2 (1.3)

AgCl and the chelators of copper and iron. Both penicillamin and bipyridyl introduced in subembryotoxic amounts throughout the whole term potentiated the toxicity of AgCl, causing an increase in the post-implantational embryolethality. The toxic effect of penicillamin was evaluated as 79%, while that of bipyridyl as 55% (embryolethality taken as an index). The copper chelator penicillamin increased the frequency of hydronephrosis and cryptorchism that are the most often seen abnormalities caused by AgCl treatment.

Discussion

Copper-containing enzymes accomplish the crucial reactions of oxidation/reduction in the cells. Alterations in the enzymatic activity of COX or SOD lead to disadaptation of an organism to the existence in the oxygen-rich environment and, finally, to death. The changes in the activity of enzymes participating in the processing of collagen can bring about serious deviations in morphogenesis due to the malformation of connective tissue. Thus copper along with iron can be considered as the most important elements, the shortage of which must dramatically affect embryogenesis.

Having the possibility to exclude completely the active copper-containing CP from the bloodstream by feeding rats on a silver salt-supplemented diet (Pribyl *et al.* 1989), we decided to study the effect of CP deficiency upon embryogenesis, since an organism in prenatal development is more sensitive both to various metabolic and toxic interventions. Such an experimental model within a short time allows us to reveal CP deficiency and its immediate consequences.

The results obtained show that CP deficiency causes severe alterations of embryogenesis which lead both to sharply increased embryolethality and to the total death of the litter. The likely explanation of the early postnatal death is the observed drop of SOD activity in the embryonic tissues. SOD deficiency seems to allow the lethal damage of tissues upon their contact with oxygen shortly after birth, i.e. the beginning of respiration is fatal for such organisms, not protected adequately against the oxygen radicals.

The above-described experiments showed the strongest embryotoxic effect of silver salts with no visible influence of those upon the pregnant females. Our suggestion that the embryotoxic effect of AgCl is caused not by the silver ions themselves, but by the diminished or fully inhibited transport of copper to the embryos due to the deficiency of CP is in good agreement with the data of another group who used laboratory animals with a mutation causing a sharp decrease of copper in the organism of pregnant and lactating females (Hurley *et al.* 1980). Apart from the high levels of embryolethality and early postnatal death, the progeny of such females had typical abnormalities of development, i.e. aneurisms of heart and aorta, underdeveloped lungs (which are symptoms of altered collagen and elastin biosynthesis (Hurley *et al.* 1980, Carnes 1971), malformations of the skeleton and neural lesions caused, particularly, by insufficient myelinization of axons (Harris *et al.* 1980). The frequency of such abnormalities could be considerably decreased if copper salts were added to the diet of pregnant and lactating females (Theriault *et al.* 1977). The role of CP or other possible carriers of copper was not studied.

At the beginning of our study the possibility of a direct effect of silver salts upon placental or embryonic tissues, e.g. by binding to sulfhydryl groups, was not excluded, although it was regarded as not very likely due to the low solubility of AgCl. However, to test the proposed mechanism of the embryotoxicity of silver we used human CP to protect the embryos from severe damage caused by AgCl. Human CP appeared to be capable of replenishing the shortage of copper-containing CP in pregnant female rats,

thus diminishing the embryotoxic effect caused by silver salts. Injections of human CP caused an elevation of copper content in placental and embryonic tissues, an increase of SOD activity in those tissues and a considerable decrease of the postnatal mortality.

It seems likely that the protective effect of the native copper-containing CP can be explained by performing its copper-transporting function. Our results are in good agreement with those obtained by another group (Campbell *et al.* 1981), who demonstrated the ability of CP to donate copper both to healthy and malignant rat tissues. In our case we can only speak of the CP-mediated copper transport across the placental barrier. Most likely, CP donates copper to other carriers in the placental tissues, which provide some kind of copper depot and also transfer the element across the barrier. Nothing can be definitely suggested about the following fate of copper in the embryo. The embryonic CP does not seem to play a role of importance in copper transport, since its concentration in the developing fetus is rather low.

The results obtained in our study show that, along with CP-mediated copper transport, there exist other mechanisms of transfer of this element across the placental barrier. This can be concluded from the fact that even with the totally inhibited enzymatic activity of CP in the plasma of pregnant rats, not all of their embryos are subjected to prenatal death and the sharp decrease of copper content in the embryonic tissues still does not result in the loss of activity by COX. Alternative (non-CP-mediated) copper transport can be accomplished by albumin or by metallothionein circulating in the bloodstream (Bremner *et al.* 1987). Indirect evidence in favor of non-CP-mediated copper transport was also obtained upon treatment of AgCl-fed rats with penicillamin. Such treatment considerably increased the embryotoxicity, while if the latter were dependent on the direct toxic effect of silver, penicillamin, the strong chelator, would have protected the organism. Synergism in the action of AgCl and penicillamin is indicative of chelating of the residual copper in the blood plasma, which strengthens the effect of copper shortage as its transport accomplished without CP is also inhibited. The proposed mechanism is also confirmed by the fact that penicillamin, introduced along with AgCl, increases the frequency of damage typical in the embryos of the AgCl-treated rats i.e. hydronephrosis and cryptorchism.

The most important result of the investigation, as we see it, is obtaining direct evidence that CP plays a role as an efficient copper carrier, at least in the period of pregnancy. The well-known phenomenon of the increase of CP biosynthesis and its plasma concentration in humans during pregnancy (Dokumov 1968) is, therefore, caused by the necessity of enhanced copper transportation to the developing embryo.

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