Assessment of Nephrotoxicity in the Chick Embryo: Effects of Cisplatin and 1,2-Dibromoethane

(mesonephros development / nephrotoxicity / cisplatin / 1,2-dibromoethane / chick embryo)

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Abstract. Morphological symptoms of mesonephric kidney damage were analysed in chick embryos treated with nephrotoxic agents – CDDP or DBE. The drugs were administered intraamniotically on ED 3 at doses 0.03 and 0.3 μg CDDP or 100 and 300 μg DBE per embryo. Body weight and absolute and relative measures of the mesonephroi (length, weight and form) were evaluated on ED 10. The higher doses of both agents affected the mass of this organ significantly. Simultaneously, a dose-dependent increase of renal malformations was detected in treated embryos, while the incidence of gross and cardiovascular defects was low (DBE) or absent (CDDP). Together with less pronounced effects on the total body growth, the results gave evidence for a higher sensitivity of the mesonephros to toxic insult when compared to the whole organism. A direct cytotoxic effect multiplied by concomitant injury of blood supply seemed to be the main cause of CDDP nephrotoxicity. In the case of DBE, damage to the mesonephros was probably associated with a primary impairment of the vascular network. The chick embryo *in ovo* provides a promising system for the assessment of nephrotoxic effects induced by prospective therapeutic agents and environmental contaminants during the prenatal period.

The embryonic kidney and liver have been shown to be especially sensitive to the action of teratogens when compared to other embryonic organs (Kavlock et al., 1982). Unfortunately, the assessment of nephrotoxic effects of xenobiotics during organogenesis does not constitute an integral or routine part of embryotoxicity testing. Kavlock and Gray (1983) introduced an *in vivo* test capable to reveal postnatal defects in kidney function after the exposure of rat dams to teratogens during a critical period of kidney development. In this system, even the treatment on embryonic day (ED) 11 (i.e. before the constitution of metanephric bud; see Shepard, 1980) was associated with postnatal dysfunction of the kidney (Kavlock and Gray Jr., 1983).

At present, however, in respect of a number of economical, ethical, juridical and scientific reasons, a programme of alternative method development is branched out with the aim to reduce the number of animals used for testing the effects of xenobiotics. New approaches, based namely upon the *in vitro* systems, give possibility to study the mechanisms of harmful effects of drugs or environmental pollutants operating at the subcellular, cellular and tissue levels. Organ and cell cultures prepared from the mouse metanephric kidney have been already applied to the study of nephron induction and molecular events participating in nephron morphogenesis. It has been assumed that culture techniques could provide a suitable tool for testing nephrotoxic effects of xenobiotics during the prenatal period (Saxén and Lehtonen, 1987). The major limitation of these *in vitro* systems, however, is their short culture period and the absence of a blood supply. This prevents a complete differentiation of nephrons and their function. In addition, the separation of organ anlage from the organism excludes the action of control mechanisms operating at the tissue and organism levels.

The chick embryonic kidney, the mesonephros, appears to be a suitable *in vivo* model for studies of renal damage induced during the embryonic period. It originates in parallel with the axis structures of the embryo already about ED 2 (Lillie, 1952; Friebová, 1975a). Urine production and other renal functions begin immediately after the completion of the blood supply to the glomeruli of the first nephron population between ED 4 and 5 (Romanoff and Romanoff, 1967; Zemanová et al., 2002). Differentiation of mesonephric nephrons continues until ED 7. Around ED 10–11, sporadic degeneration of particular nephrons has been observed (Lillie, 1952). Despite that, the growth of the majority of nephrons continues until ED 14 when the length and total weight of the organ reaches its maximum (Lillie, 1952; Friebová, 1975b). The functional maturity of the

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Abbreviations: CDDP – cisplatin, CDT – cystically dilated tubules, DBE – 1,2-dibromoethane, ED – embryonic day, GST – glutathione-S-transferase, SH – sulfhydryl, VDRT – ventrodorsally rearranged tubules.

chick mesonephros, however, is reached already between ED 9–11, as evidenced by biochemical and histochemical markers, glomerular filtration, and ion transport (Friebová-Zemanová, 1981; Clark et al., 1993; Jirsová and Zemanová, 1998). Hence, the chick mesonephros is less transitory in comparison with mammalian embryos and persists as a fully functional kidney throughout nearly half of embryonic development.

We decided to verify the suitability of the chick mesonephros for assessment of prenatal nephrotoxicity using two compounds differing markedly in chemical nature and their mechanisms of action. The first compound, cisplatin [cis-diamminedichloroplatinum (II), CDDP], is a widely used anti-cancer drug with a well-known embryotoxic and nephrotoxic effects (Madias and Harrington, 1978; Dobyan et al., 1981; Keller and Aggarwal, 1983; Hartmann et al., 1999; Ikeda et al., 1999). Its cytostatic properties are associated with highly reactive intermediates arising by hydrolysis of CDDP in aqueous solution. These intermediates are capable of forming intra-strand cross-links in DNA. RNA and proteins represent other targets of CDDP action (Eastman, 1990; Rosenberg and Sato, 1993; Leibbrandt et al., 1995). The nephrotoxicity of heavy metals (e.g. mercury) is, in general, attributed to a depletion of intracellular glutathione or direct interaction with sulfhydryl (SH) groups of proteins necessary for enzyme function. This mechanism seems to function also in the case of CDDP because the kidney of exposed rats exhibited the decline of SH groups and mitochondrial dysfunction through the impairment of glutathione peroxidase (Levi et al., 1980; Sugiyama et al., 1989; Brady et al., 1990). The role of free radicals in CDDP-induced nephrotoxicity was also hypothesized on the basis of alleviated response of the kidney to CDDP administered simultaneously with selenium (Sugiyama et al., 1989). Irrespective of the character of the insult, interference with the vital cell functions may result in the death of the cells. While high concentrations of CDDP led to necrotic cell death within a few hours, low concentrations led to apoptosis over several days (Lieberthal et al., 1996).

In mice, despite the severe embryolethal influence of CDDP, neither external nor internal malformations appeared more frequently in transplacentally exposed embryos than in a control population (Kopf-Maier et al., 1985). Similarly, intraamniotic treatment of chick embryos with CDDP between ED 2 and 4 induced a dose-dependent embryolethality without apparent teratogenic effect (Zemanová et al., 1989). Evaluation of nephrotoxicity performed two days after the intravenous administration of CDDP to chick embryos on ED 7, 11 or 14 revealed a significant increase of uric acid concentration in serum in the treated group (Zemanová et al., 1989).

The second tested compound, 1,2-dibromoethane (or ethylene dibromide; DBE), also exhibits prominent nephrotoxicity in adult organisms (Humpreys et al., 1999). In the past it was widely used as a pesticide and soil fumigant. Then, the application of DBE in agriculture has been considerably reduced due to its toxic, mutagenic and carcinogenic properties (Brem et al., 1974; Tezuka et al., 1980; Shimada et al., 1990). Nevertheless, in Australia it has been used for eradication of Papaya fruit fly as recently as the past decade (Imming, 1995). Monitoring concentrations of toxic air pollutants in Minnesota revealed for 19 of 31 substances an underestimation of modelled values when compared to actual measurements. In the case of DBE, the actual concentrations exceeded modelled values by ca 80% (Pratt et al., 2000).

In contrast to cisplatin, the harmful effects of DBE are mediated by products of metabolic biotransformation of the parental compound. The highly toxic 2-bromoacetaldehyde arises from DBE by microsomal oxidation via cytochrome P450 (Guengerich et al., 1981; Wormhoudt et al., 1996). The second, non-oxidative pathway, catalyzed by glutathione-S-transferase (GST), involves a direct conjugation of DBE with glutathione and produces a highly reactive episulphonium ion forming adducts with DNA (Ozawa and Guengerich, 1983; Guengerich, 1994). Glutathione conjugates of DBE may be transformed into cysteine conjugates, processed subsequently by renal enzymes to cytotoxic thiols or sulphoxides (Sausen and Elfarra, 1990). The ability of the kidney to process liver metabolites of DBE to even more harmful products could explain the high nephrotoxic potential of DBE.

A teratogenic effect of DBE has also been reported. For example, in rat embryonic cultures, the simultaneous addition of DBE with GST from adult individuals induced injury of the central nervous system, eyes, olfactory system and extraembryonic circulation system in both yolk sac and allantois (Mitra et al., 1992). Similar effects have also been observed after exposure of rat embryos *in vitro* to DBE alone (Brown-Woodman et al., 1998). Neither of these *in vitro* studies allowed an assessment of the long-term, adverse effects of DBE, including alterations in kidney morphogenesis.

We therefore evaluated several quantitative parameters characterizing the growth and morphogenesis of the mesonephroi in 10-day-old chick embryos exposed to a single dose of DBE or CDDP on ED 3. The analysis was completed by an evaluation of the changes in the development of nephrons – i.e. in their size and structural arrangement. The results were correlated to the occurrence of other malformations and general growth of embryos with the goal of determining any higher sensitivity of the embryonic kidney to the action of xenobiotics.

Material and Methods

Drug administration

Fertilized eggs of outbred Grey Leghorn breed (Koleč farm, Institute of Molecular Genetics, Academy

of Sciences of the Czech Republic, Czech Republic) were incubated in horizontal position at 37.3 ± 0.3˚C and 50% humidity. On ED 3 embryos at developmental stages HH 18–20 (according to Hamburger and Hamilton, 1951) received intraamniotic injections over the right lateral side of the embryo (Jelínek and Peterka, 1981) with either CDDP or DBE (Sigma-Aldrich, Prague, Czech Republic). In the case of CDDP, 0.03 and 0.3 µg were selected on the base of previous experiments as effective doses with low and high embryotoxic potential in the chick embryo (Zemanová et al., 1989). Experimental groups of embryos were exposed to 5 µl of CDDP solution in distilled water (fresh solution was prepared before each experiment), controls received the same volume of distilled water. To enhance the safety of solution preparation of the relatively volatile DBE, the compound was dissolved in 6% ethanol using the Wheaton glass serum bottles (Sigma-Aldrich) sealed by aluminium caps (tear-out center disk and teflon-faced rubber septum, Sigma-Aldrich). Before administration, the resulting emulsions were drawn several times through the septum using a gas-tight syringe with a needle. Then, 5 µl of the emulsion containing 100 or 300 µg of DBE were injected intraamniotically as described above using a gas-tight Hamilton syringe. These doses also induced a weak and a strong embryotoxic effect in chick embryos in previous experiments (Dušek et al., 2001). The control group was treated with 5 µl of 6% ethanol alone.

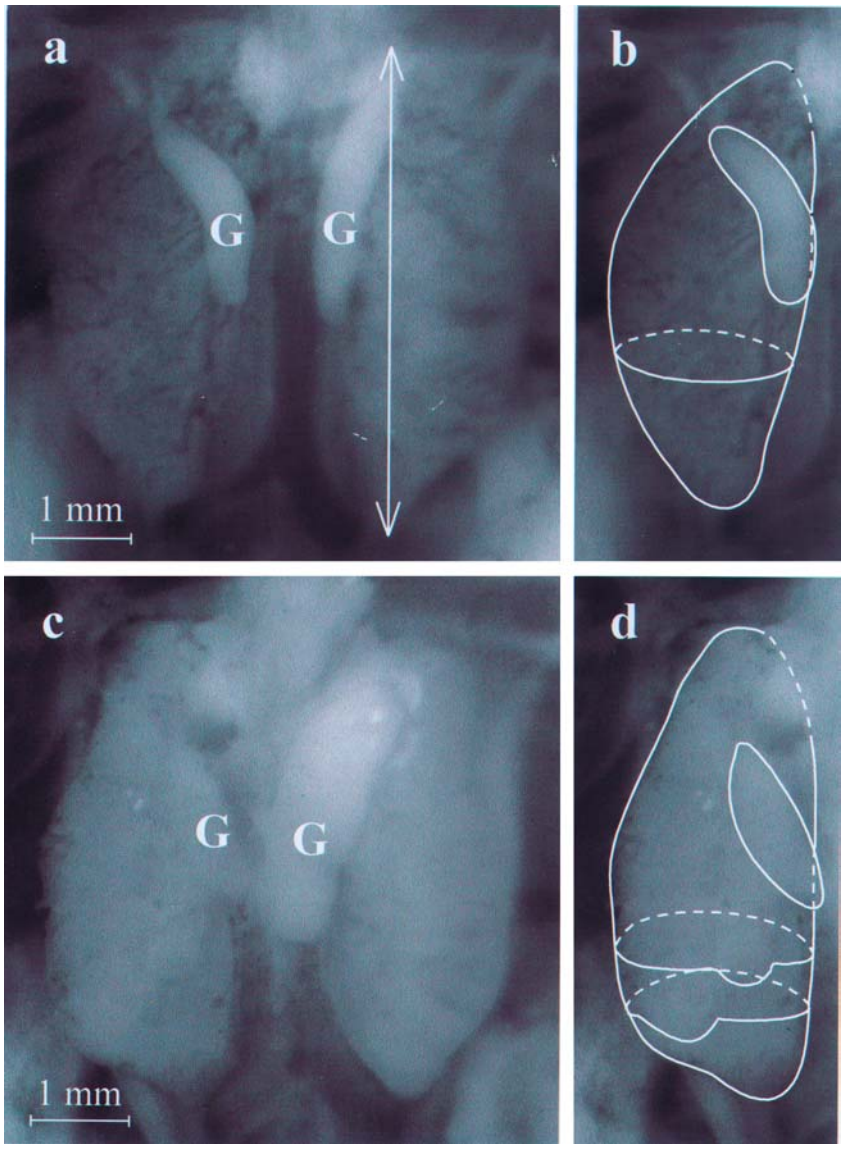

Fig. 1. Ventral aspect of 10-day mesonephroi
a) Normal form of male mesonephroi, arrow shows the length of the organ measured by the ocular micrometer. b) Highlighted contour of the gonad (G) and right mesonephros with depicted cross-section demonstrating a normal ventro-dorsal dimension. c) Mesonephroi from female embryo treated with 300 µg of DBE on ED 3 manifesting ventro-dorsal rearrangement of tubules in the right kidney. d) Highlighted contour of the gonad (G) and affected kidney with depicted cross-sections indicating the area of abnormal form of the organ.

Embryo examination and subsequent assessment of the mesonephroi

After treatment, incubation of eggs continued under standard conditions with a daily assessment of embryonic mortality. To judge short-term effects of CDDP, 24 h after each treatment, the length of embryos and the size of allantois were measured in a group of 12 treated or control embryos utilizing a stereomicroscope outfitted with a calibrated eyepiece micrometer (Dušek et al., 2001). The experiment was terminated on ED 10, when all nephron populations of mesonephros were completely structurally differentiated (Friebová, 1975a). Survivors were removed from the eggs, weighed and observed for external malformations. The trunks were then dissected for examination of the cardiovascular system with special attention devoted to the mesonephroi. The results of our visual evaluation of the mesonephric shape (arrangement of nephrons) and of the size of tubular lumina in superficial tubules were categorized using several criteria. Findings of mesonephroi with irregular clustering of nephrons in the ventro-dorsal directions were denoted as mesonephroi with ventro-dorsally rearranged tubules (VDRT; Fig. 1). All tubules exhibiting two-times or greater enlarged luminal diameters were classified as cystically dilated tubules (CDT; Fig. 2b). Several categories of CDT, including solitary, dispersed and

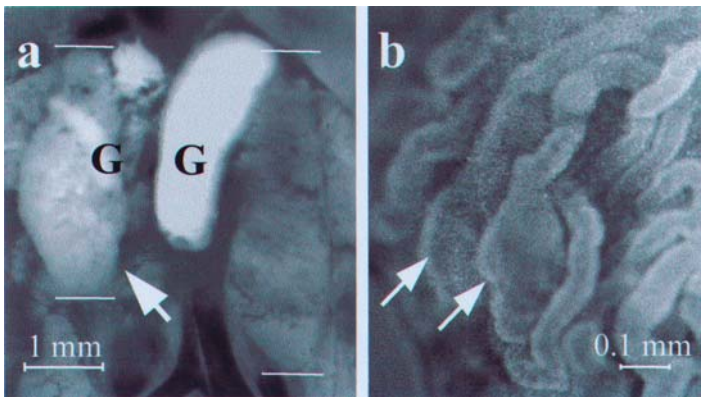

Fig. 2. Other abnormalities of mesonephros in the chick embryos
a) Extreme retardation of the growth of the right kidney in length (arrow) in a 14-day female embryo treated with 300 μg of DBE on ED 3. Horizontal lines mark the length of both mesonephroi. b) Cystic dilations of proximal tubules (see arrows) in mesonephros of a 10-day embryo treated with 0.3 μg of cisplatin on ED 3. For better visualization of tubules, mesonephros were stained 8 min with 0.01% solution of acridine orange in Ringer's buffer.

clustered CDT, were classified according to the frequency of CDT in any of the three thirds of mesonephroi; these regions were demarcated into anterior, medial, and caudal thirds oriented perpendicular to the body axis. Then, the lengths of both mesonephroi were measured separately under a stereomicroscope (Leica MZ6, Leica Microsystems Wetzlar GmbH, Germany) with an eyepiece micrometer (Fig. 1a). Finally, the whole-paired organ was taken out and weighed. From these basic parameters we derived two quantitative parameters that easily characterized deviations in the mesonephric development. The first, the relative weight of mesonephroi, calculated as a proportion of the total weight of both mesonephroi to the weight of the whole embryo, reflects the growth retardation of mesonephroi. The second parameter, a normalized weight of mesonephros (i.e. the weight of 1 mm of the mesonephros length), was calculated from the average weight of mesonephros related to the average length of the organ. This parameter characterizes the growth of the nephron tubules in a unit portion of the mesonephros, since nephrons are monotonic, i.e. independent of their location on the longitudinal axis of the organ.

Statistical analysis

The homogeneity of data on mesonephros length and weight as well as on the weight of embryos was tested by two-way analysis of variance. The significance of differences between the treated and control groups was evaluated by the Student-Newman-Keuls multiple range test. The increase in the incidence of embryos with malformed mesonephroi after the exposure to each compound was tested using the χ^2 test. The differences between the exposed and control group in the values of relative and normalized weight of mesonephroi as well as the length of the whole embryos were tested by the t-test. Changes in allantois size were analysed using the paired t-test.

Results

Cisplatin

Within 24 h after the treatment with 0.3 μg of CDDP the embryos exhibited a mild growth retardation in comparison with control individuals (10.93 ± 0.91 mm and 11.58 ± 1.06 mm, respectively). Simultaneously, a severe anaemia and impairment of the vascular network in the yolk sac and allantois were observed in the group of treated embryos, which corresponds with our previous findings (Dušek et al., 2001). In addition, the treated group exhibited a significant delay in allantois growth ($P < 0.01$).

Embryotoxic effects of CDDP recorded on ED 10 are summarized in Table 1. The low dose of 0.03 μg of CDDP did not influence the survival of embryos, while the high dose of 0.3 μg of CDDP resulted in the death of ca 52% of exposed embryos. The teratogenic effect was low in both treated groups – the incidence of survivors with external malformations and cardiovascular defects reached at maximum 8.3% and did not differ substantially from the controls. A sporadic occurrence of anophthalmia, hypoplasia of maxilla and mandibula, abdominal eventration, a defect of the interventricular septation of the heart and pygostyle reduction were observed in the treated embryos. The controls exhibited only the pygostyle reduction. In contrast, a dose-dependent response was observed in the incidence of mesonephric malformations. Solitary CDT and VDRT were detected almost exclusively in both treated groups (Table 2). The high dose of CDDP reduced simultaneously the absolute length and weight of the mesonephroi as well as the weight of the whole embryos (Table 3). Further, the relative mesonephric weight decreased significantly only after the treatment with the high dose of CDDP (Table 4). Both groups of treated embryos exhibited a dose-dependent reduction of the normalized weight of the mesonephroi (Table 4).

1,2-Dibromoethane

Embryotoxic effects of DBE are summarized in Table 5. Embryolethality increased considerably with the given dose of DBE. The teratogenic effect showed a similar, though less pronounced, effect. Embryo malformations included brain abnormalities (hydrocephaly, exencephaly), cardiovascular defects (stenosis of aorta, missing aorta, truncus communis), and amniotic bands; microphthalmia and pygostyle reduction were also detected occasionally. Only pygostyle reductions appeared in controls. The low dose of DBE (100 μg) induced a 2.4-fold rise in the incidence of embryos with malformed

Table 1. Embryotoxic effects of cisplatin

Dose [μg]	N[a]	D[b] [%]	Teratogenic effect[c] [%]	Malformed M[d] [%]
0	41	9.8	5.4	0
0.03	39	7.8	8.3	17*
0.3	95	51.6	6.5	30**

[a]N, number of treated embryos; [b]D, number of dead embryos; [c]number of embryos with external malformations and cardiovascular defects; [d]number of embryos with malformed mesonephros; * $P < 0.05$; ** $P < 0.01$

Table 2. Malformation spectrum of mesonephros after the treatment with cisplatin[a]

Type of malformation	Control embryos	0.03 μg	0.3 μg
Solitary CDT[b]	0	11.1	19.6
Dispersed CDT[c]	0	0	2.2
Clustered CDT[d]	0	0	0
VDRT	0	11.1	10.9

[a]Data represent the percentage of survivors with a given malformation (see Table 3); [b]1-3 CDT in one third of mesonephros; [c]4-5 CDT in one third of mesonephros; [d]more than 5 CDT in one third of mesonephros

Table 3. Nephrotoxic effects of cisplatin[a]

Dose (μg)	S[b] (%)	Weight of embryo (mg)	Length of M[c] (mm)	Weight of M[d] (mg)
0	90	2461 ± 229	5.14 ± 0.37	8.6 ± 1.5
0.03	92	2360 ± 280	5.08 ± 0.43	7.9 ± 1.9
0.3	48	2295 ± 263**	4.87 ± 0.41**	6.7 ± 1.5**

[a]Data represent mean ± standard deviation; [b]S, number of survivors; [c]average length from both mesonephroi; [d]total weight of both mesonephroi; ** $P < 0.01$

Table 4. Growth retardation of the mesonephros and the nephrons in embryos treated with cisplatin or 1,2-dibromoethane (source data see in Tables 3 and 7)[a]

Cisplatin			1,2-Dibromoethane		
Dose(μg)	Relative weight of M[b] (μg/mg)	Normalized weight of M[c] (mg)	Dose (μg)	Relative weight of M[b] (μg/mg)	Normalized weight of M[c] (mg)
0	3.43 ± 0.63	0.84 ± 0.16	0	3.37 ± 0.69	0.78 ± 0.16
0.03	3.33 ± 0.83	0.77 ± 0.17*	100	3.44 ± 0.70	0.77 ± 0.16
0.3	2.93 ± 0.61 **	0.69 ± 0.13**	300	2.92 ± 0.64**	0.69 ± 0.15 *

[a]Data represent mean ± standard deviation; [b]weight of both mesonephroi/weight of embryo; [c]weight of both mesonephroi/length of both mesonephroi; * $P < 0.05$, ** $P < 0.01$

Table 5. Embryotoxic effects of 1,2-dibromoethane

Dose (μg)	N[a]	D[b] (%)	Teratogenic effect[c] (%)	Malformed M[d](%)
0	38	23.7	6.6	13.8
100	28	35.7	11.1	33.3
300	127	70.9	18.9	51.4**

[a]N, number of treated embryos; [b]D, number of dead embryos; [c]number of embryos with external malformations and cardiovascular defects; [d]number of embryos with malformed mesonephros; ** $P < 0.01$

mesonephroi, while the general teratogenic effect (the external malformations and cardiovascular defects) increased only 1.7 times compared to controls. Statistical analysis showed a significant increase in the number of embryos with malformed mesonephroi only after the administration of the high dose (300 μg) of DBE, probably due to the sporadic occurrence of the solitary CDT in the control group. Nevertheless, the frequency of the solitary

Table 6. Malformation spectrum of mesonephros after the treatment with 1,2-dibromoethane[a]

Type of malformation	Control embryos	100 µg	300 µg
Solitary CDT[b]	13.8	22.2	37.8
Dispersed CDT[c]	0	5.6	5.4
Clustered CDT[d]	0	0	2.7
VDRT	0	5.6	18.9

[a]Data represent the percentage of survivors with a given malformation (see Table 7); [b]1-3 CDT in one third of mesonephros; [c]4-5 CDT in one third of mesonephros; [d]more then 5 CDT in one third of mesonephros

Table 7. Nephrotoxic effects of 1,2-dibromoethane[a]

Dose (µg)	S[b] (%)	Weight of embryo (mg)	Length of M[c] (mm)	Weight of M[d] (mg)
0	76	2390 ± 174	5.17 ± 0.35	8.0 ± 1.7
100	64	2238 ± 223	5.04 ± 0.36	7.5 ± 1.3
300	29	2373 ± 155	5.03 ± 0.47	7.0 ± 1.5*

[a]Data represent mean ± standard deviation; [b]S, number of survivors; [c]average length from both mesonephroi; [d]total weight of both mesonephroi; * $P < 0.05$

CDT was substantially higher in both groups of treated embryos. All other types of mesonephric malformations were detected only in DBE-treated embryos (Table 6). The weight of embryos did not change significantly after treatment with DBE (Table 7). In contrast, the weight and length of the mesonephroi indicated a negative dose-response – i.e. a decrease with increasing dose of DBE (Table 7). The assessment of relative parameters confirmed a significant reduction of both, the relative and normalized weight of the mesonephroi, only after the administration of the high dose of DBE (Table 4). Mesonephros shortening as well as structural rearrangements (VDRT) registered on ED 10 persisted to later developmental stages despite the proceeding growth of mesonephros (Figs. 2a, 3).

Discussion

Both tested compounds interfered with the morphogenesis of chick embryo mesonephroi. Cisplatin induced a dose-dependent increase of mesonephric malformations manifested as solitary CDT and spatial rearrangements of the tubules, while the external morphology of the embryos, as well as the cardiovascular system exhibited no remarkable changes. A lack of gross developmental abnormalities and strong embryolethality corresponded to the findings in laboratory rodents (Keller and Aggarwal, 1983; Kopf-Maier et al., 1985). A significant reduction of the mesonephric weight and length was noted only after the administration of 0.3 µg of CDDP. This effect was accompanied by a simultaneous reduction in the total weight of embryos, which suggested that the observed nephrotoxicity may be an integral part of the general growth retardation. The decline of the relative weight of the mesonephroi confirmed, however, a real growth retardation of the embryonic kidney itself. This finding was supported by a significant decrease of normalized mesonephroi weight, reflecting a diminished growth of the mesonephric nephrons in length and a likely decrease in the number of nephron populations. In the

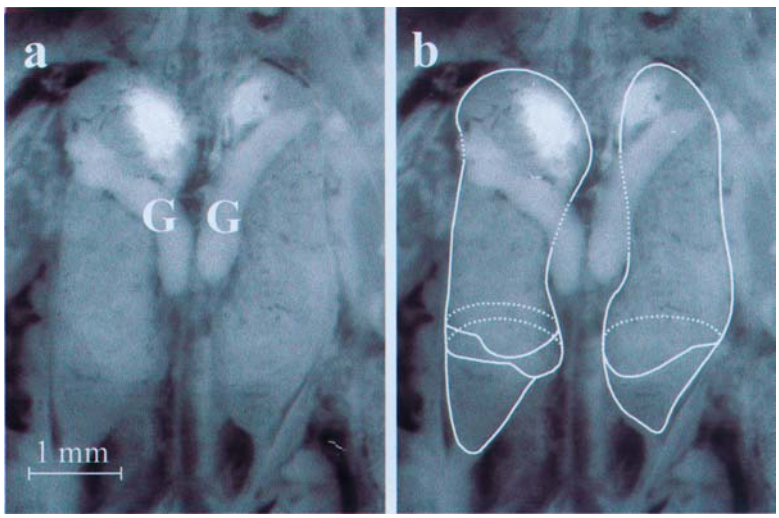

Fig. 3. Ventral aspect of 12-day mesonephroi from a male embryo treated with 300 µg of DBE on ED 3 manifesting bilateral ventro-dorsal rearrangement of tubules.
a) Native view. b) Highlighted contour of the mesonephroi with depicted cross-sections indicating the area of abnormal form of the organ.

chick mesonephros, nephron tubules are situated perpendicularly to the long axis of the organ and their growth determines the final width of the mesonephros. Newly formed populations of nephrons are added in the ventro-dorsal direction to the older ones along the whole mesonephros length. A significant reduction of the normalized weight of the mesonephroi unambiguously documented a reduction in tubular growth, even in embryos exposed to the low dose of CDDP, which exhibited no remarkable embryolethality.

In young rats, only the highest dose of CDDP (from the tested range 1.5–6 mg/kg/day) induced nephrotoxic responses such as oliguria, proteinuria and a reduction of p-aminohippurate excretion (Appenroth and Bräunlich, 1984). A dose 2.88 mg/kg of CDDP represented the LD_{50} for rat embryos exposed transplacentally on ED 6 – i.e. during the implantation period. During organogenesis (ED 11), this value decreased to 1.0 mg/kg (Keller and Aggarwal, 1983). In our experiments, the LD_{50} for 3-day-old chick embryos corresponded to the high dose of CDDP representing approximately 2.3 mg/kg of the living mass (i.e. including extraembryonic tissues). The nephrotoxic effect, manifested by a significant diminution of the morphological parameters of the mesonephroi and by an increase in cystic dilations was detected, however, even with the 10-fold lower dose of CDDP. The cytotoxic effect resulting from the interaction of CDDP with DNA and/or SH groups of proteins is undoubtedly responsible for the observed impairment of mesonephros development as well as for the total growth retardation in the chick embryo. Malnourishment of embryonic tissues in consequence of the damage to the blood supply then likely potentiates the direct toxic effects of the compound. The extreme sensitivity of the kidney of adult organisms to the toxic insult is usually attributed to a high concentration of the toxic products in the organ due to its excretory function. A similar mechanism could contribute to the nephrotoxic effects of CDDP in the chick embryos. In addition, even the very early development of the mesonephroi is closely dependent upon appropriate vascularization. Damage to the vascular network may therefore also play an important role in dysmorphogenesis of mesonephros. The occurrence of a variety of cystic dilations gives evidence for impaired resorption function in proximal tubules of these mesonephroi, which could be associated with a delayed maturation of the nephrons after the initial cytotoxic injury.

In the case of DBE, no growth retardation of either the whole embryo or mesonephros was observed after the treatment with 100 μg of the compound. This was in spite of a mildly increased incidence of gross developmental abnormalities and mesonephric malformations. In contrast, the high dose of DBE (300 μg), in addition to exhibiting stronger teratogenicity, significantly affected the growth of the mesonephroi, although the nephrotoxic effect was less pronounced in comparison with CDDP. Also in contrast to CDDP, not even the high dose of DBE affected the total growth of embryos significantly. This suggests that damage to the blood supply of kidney might be responsible for DBE-induced dysmorphogenesis of the mesonephros. Indeed, the study of acute effects of DBE in the chick embryos revealed a severe disturbance of the vascular network at the site of administration. This was manifested as a haemorrhage in the vascularized region of hindbrain and massive bleeding from the anterior vitelline vein into amniotic sac, which likely represented a major cause of extensive embryonic mortality (Dušek et al., 2001). In agreement with the findings in the rat embryonic cultures (Mitra et al., 1992; Brown-Woodman et al., 1998), 24 h after the treatment the chick survivors exhibited a reduced vascularization of the extraembryonic membranes. The origin of brain malformations as well as of aortal defects and amniotic bands in the parietal region was apparently associated with the primary impairment of the vascular network at the site of DBE administration (Dušek et al., 2001).

In contrast to rodent embryos, the chick embryo is capable of biotransformation of xenobiotics even from very early stages of development. Before the constitution of a functional liver on ED 5, the metabolic processes are mediated by enzymes localized in the yolk sac (Gamett and Klein, 1984). However, the character of DBE-induced changes in the chick embryo seems to correspond rather to the direct effects of the parental compound on morphogenesis, which were reported in rat embryonic cultures (Brown-Woodman et al., 1998). The interaction of DBE with cell membranes could affect the viability of the cells either directly, by means of damage to transport functions, or indirectly, through impaired vascularization of the yolk sac and embryonic organs; this would result in malnourishment of embryonic tissues. The contribution of DBE metabolites to the final embryotoxicity in the chick embryo, however, cannot be excluded because of their high embryotoxic potential. Using rat embryos *in vitro*, quite comparable results were obtained after a long exposure to DBE and after a short exposure combined with the addition of GST (Mitra et al., 1992; Brown-Woodman et al., 1998). On the assumption that DBE metabolites are distributed by the blood stream to the embryo, their extremely short half-life (Inskeep et al., 1986) could explain the prevalence of the early effects of DBE near the site of administration to the chick embryo.

The higher tested dose of DBE, 300 μg, resulted in the death of about 70% of embryos treated on ED 3 – in agreement with previously published data (Dušek et al., 2001). It is, however, impossible to simply compare our results with the dose of DBE effective in rat embryos *in vitro.* While embryonic cultures represent a closed system where the organism may be exposed to the exact volume of the tested compound for a defined time period,

the chicken embryo developing in egg is a complex system interacting with its environment. Here, the parental compound is very quickly distributed from the place of administration to the extraembryonic tissues and yolk because of patent circulation and the muscular contractions of the amnion itself. Within 6 h after the administration only about 0.1% of the original compound was detected in the amniotic sac with the embryo inside, and 77% was found in the yolk and extraembryonic tissues (unpublished results). Nevertheless, a certain amount of DBE was found in the embryonic tissues even 48 h after the administration, suggesting a continuous exposure to the parental compound. The contribution of the extraembryonic metabolism to the rapid decline of DBE concentration in the egg remains to be clarified.

Our experiments showed a high sensitivity of the chick mesonephroi to the action of both tested compounds. In comparing their molar concentrations, CDDP appeared to be 1000 times more effective than DBE. Such comparisons are problematic, however, due to the poor solubility of DBE, which had to be administered in emulsion while CDDP was used in solution. The results suggest that the nephrotoxic effects of CDDP in the chick embryo could result from the cytotoxic injury of the developing mesonephros potentiated by the concomitant damage to mesonephros vascularization. On the other hand, the nephrotoxicity of DBE is probably associated, above all, with the primary impairment to the vascular network.

The mesonephros functions as an early excretory organ long before the development of the definitive, metanephric kidney. Also in contrast to the definitive kidney, the oldest mesonephric nephron population resides permanently on the ventral surface of the organ, making them exquisitely accessible to observation and measurement. The choice of this transitory kidney thus significantly reduces the time required for an evaluation of early renal effects and enables continuous observation of the developing nephrons. In addition, a portion of the mesonephros persists in male embryos through the foetal period and postnatally transforms – comparable with mammals – into epididymis. Damage to the mesonephroi could therefore disrupt later reproductive function.

The chick embryo *in ovo,* particularly its mesonephros, represents a suitable and prospective alternative model for prenatal nephrotoxicity testing.

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