Methods for Assessing the Effects of Chemicals on Reproducting Functions
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Methods for Evaluating and Predicting Human Fetal Development

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1. INTRODUCTION

Whenever a pregnant woman is exposed to infectious agents, ionizing radiation or chemicals, including drugs, there is always a possibility that such exposures may have adverse effects on the development of the fetus. A therapy that may be of great benefit to the sick mother, may be at the same time hazardous to the fetus.

A teratogen may be defined as a physical, chemical or biological agent producing structural or functional abnormalities in an organism exposed to it before birth. The sensitivity of the conceptus to an exogenously induced damage varies widely depending on the stage of development (Wilson, 1972). At the early stage of ontogenesis, before blastogenesis is completed (in humans during the first three weeks after conception) the conceptus, if exposed to a noxious agent such as ionizing radiation, tends to have an "all-or-none" response (Russel and Russel, 1954). The embryo survives without any anatomical damage, or dies and is aborted. During organogenesis, approximately 4–12 weeks after conception, the sensitivity of embryo to morphologic malformations is high, the peak of sensitivity being during the fifth and sixth week after conception. The period of peak sensitivity to teratogens includes the constitution of embryochorionic circulation and early differentiation of many organs. There are "critical periods" in morphogenesis of all structures (Schwalbe, 1906; Saxén and Rapola, 1969). Adverse influences at critical periods may involve not only morphologic malformations, but may result in adverse changes of postnatal functions as well.

Possible teratogens are tested using several species of animals. In spite of similarities in the developmental pattern, the peculiarities of some species make the comparisons extremely difficult. For instance, inversion of the germ-layers in mice and rats and the resulting temporary yolk sac placenta does not occur in other mammals. Also the metabolism of chemicals in the maternal organism may differ significantly between species.

Table 1 Comparative classification of prenatal development of quadruped vertebrates

Comparative stages		Human		Monkey		Rat		Mouse	
		Length	Age	Length	Age	Length	Age	Length	Age
	Stage	in mm	in days	in mm	in days	in mm	in days	in mm	in days
Unicellular	1	0.2	0–2	0.15	1		1		1
Blastomeric (16–20 blastomers)	2	0.2	2–4	0.15	2–4		2–3		2–3
Blastodermic	3	0.4	4–6	0.2–0.3	7–9		4–5		4–4½
Bilaminar embryo stage:									
bilaminar plate	4–1	0.1	6–14	0.3	10–13		6–7		5
primary yolk sac	4–2								
secondary yolk sac	4–3	0.2–0.4							6½
Trilaminar embryo stage:									
with primitive streak	5–1	0.4–1.0	15–17	0.5	17–19		8–9		7
with a notochordal process	5–2	1.0–2.0	17–20	1.5					
Early somite stage:									
completely open neural groove	6–1	1.5–2.0	20–21	1,9	21–24		9½		8
neural tube closing, both ends open	6–2	1.5–4.0	21–26	4.0	24	1.3–3.0	10–10–3/4		

one or both neuropores closed	6–3	3–5	26–30	4.0–6.0	24–26	3–4.1	11	1.8–3	$9\frac{1}{2}$
Stage of limb development: bud of proximal extremity	7–1	4–6	28–32	6.0	26	4–4.5	11	2–3.3	$9\frac{1}{2}$–10
buds of proximal and distal extremities	7–2	5–8	31–35	8.0	27	4–6	11.5	7.9	$10\frac{1}{2}$
proximal extremity two segments	7–3	7–10	35–38			5.8–8	13		11
proximal and distal extremity two segments	7–4	8–12	37–42	7–9		8–9.5	$13\frac{1}{2}$		
digital rays, foot plates	7–5	10–14	42–44	10	34	10	14	11.5	13
digital tubercles	7–6	13–21	44–51	9–11	36	12.5	$15\frac{1}{2}$	13	14
digits, toe tubercles	7–7	19–24	51–53	19	41	16	17	14	
Late embryonal stage: differentiated extremities	8–1	22–23	52–56	22	44	19	$17\frac{1}{2}$	14–17	18
using eyelids	8–2	27–35	56–60	39–44	52	22	$19\frac{1}{2}$	22	16–18
Fetal period	9	31–200	60–182+	40–260	53–170	25	19–21	22–25	18–10
Perinatal period	10	201–450	180–266+	Post-natal	Post-natal	Post-natal			

The goal of teratogenicity tests is to determine the dose which is embryotoxic in animals causing intrauterine death or growth retardation, or which is teratogenic, producing anatomical malformations. The mechanism of damage is not often investigated. General mechanisms of interference with prenatal growth and differentiation include inhibition of cell proliferation related to altered synthesis of proteins, carbohydrates and lipids, or to limited energy supply; inhibition of placental transfer and protein synthesis; interference with fetal and fetoplacental circulation; and altered or inhibited formation of secretory products.

The type of malformation is related to the stage at which embryo is exposed to a teratogen. For instance, a human conceptus aborted during third gestational month after exposure to aminopterin (a folic acid antagonist) showed trophoblastic necrosis, thrombosis of the intervillous space and damaged fetal hematopoiesis within the liver (Shaw, 1972). If the fetus survived, a typical aminopterin syndrome was characterized by malformations in the central nervous system, craniofacial dysplasia, clubhands and clubfeet, and mental retardation (Thiersch, 1952; Howard and Rudd, 1977).

The effects of the chemicals which have been tested may be classified as expected and unexpected. The expected effects are gross malformations, abortion and clefts resulting from inhibition of growth observed after administration of aminopterin, busulfan, chlorambucil, cyclophosphamide and tolbutamid in the first trimester of pregnancy (Schiff et al., 1970). Growth retardation may result from the treatment with cortisone (Warrel and Taylor, 1968). Masculinization of female fetuses is an expected consequence of administration of androgenic substances such as testosterone and its derivatives (Grumbach and Ducharme, 1960). The interference of diethylstilbestrol (Herbst et al., 1971) with normal regression of paramesonephric epithelium within the vaginal plate leading to vaginal adenosis should be considered as a predictable effect since vagina is a known target of estrogens. Tetracyclin is known to form chelates with calcium, and after application to pregant women it is deposited in the fetal bone mineral and deciduous teeth (Toaff and Ravid, 1968). A less expected effect is the deposition of tetracycline into the lenses leading to cataracts (Harley et al., 1964). Understandable is the eighth nerve damage related to neomycin, streptomycin and quinine and the formation of fetal goiter after administration of anti-thyroid drugs during pregnancy.

Unexpected teratogenic effects are the effects which are difficult to predict even if the metabolism of the chemical is known. For instance, the phenocopy of the stippled epiphyses syndrome (or chondrodysplasia punctata) which develops after administration of dicumarol anticoagulants (such as warfarin) during the first three months is difficult to predict (Shaul and Hall, 1977). Similarly, the properties of thalidomide do not forewarn the character of the now well-known syndrome. Unexpected effects may be sometimes detected in animal experiments. However, it should always be understood that extrapolation of animal experiments to man is valid only to a limited degree.

Teratologists and experimental embryologists have provided much information on the developmental mechanisms and their disturbances in different vertebrates. To make the interpretation of results easier, we have proposed a comparative classification of prenatal development of quandrupeds in which corresponding stages are marked by the same system of numbers. The prenatal development of man, macaccus, rat and mouse is compared in Table 1.

2 MOTHER-FETUS AND FETUS-MOTHER TRANSFER; THE PLACENTAL BARRIER

The transfer of substances from the maternal into the fetal compartment takes place either across the placenta or across fetal membranes. Only under therapeutical or experimental conditions substances are administered directly into the amnion, or into the peritoneal cavity of the fetus (intrauterine fetal transfusion). Transport mechanisms are related to the anatomy of the biological barrier, to the uterochorionic and uteroplacental (maternal) circulation and to the embryo-chorionic or feto-placental (fetal) circulation.

The total maternal uterine blood flow is considered to be 94–127 ml/minute/kg at 10–28 gestational weeks and approximately 150 ml/minute/kg at the term (Assali et al., 1960). The blood flow entering intervillous space is intermittent, and the blood pressure in the terminal uterine arteries is 70–80 mmHg (Alvarez and Caldeiro-Barcia, 1950). The blood pressure in the intervillous space is approximately 10 mmHg, and increases during uterine contractions up to 30–50 mmHg. The pressure in the maternal veins leaving the intervillous space is about 8 mmHg. The blood pressure within the intervillous space is lower than that within the chorionic (fetal) vessels. The blood pressure in the umbilical arteries is approximately 48–50 mmHg and in the vein 24 mmHg (Margolis and Orcutt, 1960). These conditions favor feto-maternal transport.

The paraplacental transfer is effected by a transport from decidual cells across the chorion into the amnion. For instance, prolactine released from decidual cells crosses the chorion and amnion and diffuses into the amnionic fluid (Riddick and Masler, 1981). The transport of decidual prolactine requires active protein synthesis and an intact microtubular system. The permeability of amniochorion can be easily studied in vitro (Seeds *et al.*, 1980).

The placental transfer takes place by diffusion, by facilitated diffusion, by active transport (against the gradient), by endocytosis, or by other poorly understood mechanisms, and depends on the physicochemical properties of chemicals, such as molecular weight, ionization, lipid solubility and protein binding (Mirkin, 1976)

Compounds with low molecular weight diffuse more rapidly. If the molecular weight exceeds 1000u, the passage across the placenta is usually slow. Molecules in non-ionized state cross the placental barrier more rapidly than in ionized state. Lipid solubility facilities the transport. There is a substantial difference between protein binding in the maternal and fetal blood plasma. This difference may exert

a significant influence on the transfer of chemicals, especially if the free substance crosses the placental barrier slowly. There are some specific placental transfer systems involving specific conjugation of the substance crossing the placenta to a complex which is cleaved before the substance is released after the transport across the trophoblast.

The placenta, especially the trophoblast, contains enzymes metabolizing endogenous as well as xenobiotic substrates (Hagerman, 1969). Some of them are of fundamental physiological importance, for example, the monoamine oxidase (De Maria, 1963) which deaminates a number of substrates such as epinephrine, norepinephrine, dopamine, serotonine and some amphetamines; diamine oxidase (histaminase) (Weingold and Southern, 1968); catechol-O-methyltransferase which inactivates epinephrine and norepinephrine; several peptidases, such as cystine aminopeptidase (oxytocinase) (Mathur and Walker, 1968; choline acetytransferase (Bull *et al.*, 1961); 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, a very potent steroid arylating system, and others.

The effects of chemicals on the placental vasculature can be studied in vitro in perfusion experiment (Mancini and Gautier, 1964). Serotonin, norepinephrine, LSD and some other hallucinogens, morphine and codeine are potent constrictors of placental vessels, at least in vitro (Gant and Dyer, 1971). Prostaglandin $F_{2\alpha}$ enhances the effect of oxytocin (Jungmannová *et al.*, 1975).

It is evident that placental enzymatic systems can metabolize a large number of endogenous as well as exogenous substances. Hydrolyzable substrates are usually hydrolyzed, and vasoactive substances and hormonal peptides are metabolized to inactive substances. Placental conjugations, with a possible exception of acetylation, seem not to occur.

3 PRENATAL DETECTION OF FETAL MALFORMATIONS AND CHROMOSOMAL AND GENETIC DISEASES

Techniques used for prenatal detection of fetal disorders can be classified as invasive and non-invasive. Invasive techniques, such as amniocenthesis and fetoscopy, include penetration into the amniotic cavity. Non-invasive techniques are based on X-ray or ultrasound procedures, or on different kinds of fetal monitoring.

3.1. Procedures Based on Amniocenthesis

Amniocenthesis is usually performed at 16–18 weeks of gestation. The volume of amniotic fluid at this stage is approximately 120–300 ml, from which 5–20 ml are withdrawn and used for analysis. The risk of complications related to amniocenthesis is less that 0, 5% (Simpson and Martin, 1976). Potential maternal complications include intra-abdominal bleeding, puncture of the bladder or

intestine and Rh-immunization. Possible fetal complications are injuries to the fetus by the needle, bleeding from placental vessels, intraovular infection and abortion. Leakage of amniotic fluid has also been reported.

The amniotic fluid sample is centrifuged, and the cells are used for cytologic examination, cultivation and subsequent chromosomal and metabolic analysis. The fluid can be examined by biochemical methods.

3.1.1 Cytology of Amniotic Cells

Centrifuged cells from amniotic fluid are spread on a microscopic slide, fixed in formalin in vapor and, if an open neural tube defect is suspected, the smear is checked for the presence of glial elements (Brock, 1978). The glial elements are bipolar, elongated and detectable by a glial protein S-100 exhibiting a specific immunofluorescence (Sarkar *et al.*, 1980). Analysis for X-chromatin and Y-chromatin in uncultivated amniotic cells can be easily performed; however, the results are far less informative than karyotyping.

3.1.2 Amniotic Cells Tissue Cultures

Centrifuged cells from amniotic fluid are cultivated for approximately 2–4 weeks in a nutrient medium containing calf serum and antibiotics. The cultivation is successful in 70–99% of cases. If karyotyping is required, colchicine is added to the growing culture (mitoses are arrested at metaphase), the medium of the culture is hypotonized and acetic acid-methanol fixative added; cells are spread on microscopic slides and stained by different banding techniques. Using this method all numerical chromosomal abnormalities can be detected. The most common is the monosomy for the X-chromosome (45,X). The other quite frequently occurring pathologic karyotypes are 47,XXY; 47,XXX; 47, + 21; 47, + 18; 47, + 13.

The detection of structural chromosomal anomalies depends on the extent of chromosomal lesions. Minor abnormalities may remain undetected. Structural chromosomal abnormalities involve deletions, translocations and rearrangements resulting in the formation of isochromosomes, incomplete chromosomes, fragments, ring-chromosomes, etc. Chromosomal breaks are observed after X-ray irradiation.

The most important indication for prenatal karyotyping of the fetus are chromosomal abnormalities (such as balanced translocation or inversion) in either parent, advanced maternal age or existence of individuals affected by chromosomal aneuploidy, or another chromosomal abnormality in the family.

Cultures from amniotic fluid of fetuses with open neural tube defects contain glial cells which are elongated, bi-polar and exceptionally adhesive (rapid adherent or RA cells), and contain a specific glial protein (Sarkar *et al.*, 1980).

Cultures of amniotic cells can also be used to detect many metabolic diseases with known biochemical basis. The biochemical defect is usually identified by

enzyme assays in cultured cells. About 75 different metabolic disorders (such as mucopolysacharidoses, glycogenoses, lipidoses, aminoacidopathies, etc.) can now be diagnosed prenatally (Golbus *et al.*, 1976; Rhine, 1976).

3.1.3 Biochemical Analysis of Amniotic Fluid

(a) *Alpha-fetoprotein (AFP).* AFP is synthesized in fetal endodermal structures such as the yolk sac, liver and gut. AFP in amniotic fluid originates probably from fetal urine, and its amniotic concentration decreases with advancing gestational age (Brock and Sutchiffe, 1972). Elevated concentrations of AFP are encountered in open neural tube defects (anencephaly, open spina bifida) and have also been reported in sacrococcygeal teratoma, omphalocele and congenital nephrosis and in pregnancies with fetal deaths; elevated AFP is sometimes present also in fetuses with atresias of digestive tube, polycystic kidneys, annular pancreas, hydrocephalus, Fallot's tetralogy and congenital skin defects. Normal concentrations of amniotic AFP during gestational weeks 16–18 are between 20 and 7 ng/ml. In fetuses with open neural tube defects, the AFP values exceed 25 ng, reaching in some cases 400–500 ng/ml (Brock, 1978). Contamination of the amniotic fluid with fetal blood is the most common source of false positive results.

(b) *Hormones in amniotic fluid.* Fetal sex can be predicted from the amniotic concentration of testosterone, FSH and LH. In male fetuses, testosterone concentration is much higher, and FSH and LH concentration lower than in female.

The differences in amniotic concentrations of testosterone, FSH and LH during gestational weeks 16–18 are shown in Table 2.

Estimation of amniotic concentration of FSH and 3, 3′, 5′-triodothyronine (RT_3) could be useful for prenatal diagnosis of fetal hypotheyroidism, either congenital or induced by exposure of pregnant women to strumigens (Chopra and Crandall, 1975). Elevated amniotic 17-hydroxyprogesterone was observed in fetuses affected by adrenal cortical hyperplasia due to C-21 steroid hydroxylase deficiency (Pang et al., 1980).

Table 2 Differences in hormone levels in the fetal amniotic fluid of males and females*

Amniotic concentration during 16–18 gestational weeks	Males	Females
T	140–190 pg/ml	20–30 pg/ml
FSH	0.05–0.12 ng/ml	0.5–0.7 ng/ml
LH	3–4 ng/ml	9–12 ng/ml

* Data from Clements *et al.*, 1976; Dawood and Saxena, 1977; Jirasek *et al.*, 1980.

3.2 Fetoscopy

Direct visual observation of the fetus is performed by a fetoscope, a special fiberoptic endoscope. Although simultaneous visualization of the entire fetus is not possible, isolated portions of the fetus such as scalp, eyes, lips, fingers, toes, external genitalia and the anterior body wall, including the insertion of umbilical cord, may be directly examined. In some cases the visualization of a structure, whose anatomy is to be examined may be very difficult because of the contamination of amniotic fluid either by meconium or by blood. In our hospital, fetoscopy is performed under general anesthesia. The Wolf fetoscope (3, 2 mm diameter) is inserted into the uterus under visual control after a small laparotomy has been performed; a suture is inserted around the fetoscope insertion. The localization of the placenta is always determined ultrasonically and the transplacental route of fetoscope insertion is strictly avoided. The risk of fetal death and abortion after fetoscopy is approximately 20%. Abortions are related to leakage of amniotic fluid and subsequent amniochorionitis, rarely to intra-amniotic bleeding. Chronic leakage of amniotic fluid during the third trimester requires a long-term hospitalization in approximately 30% of patients. Fetoscopy allows prenatal diagnosis of all major external morphological malformations such as facial clefts, deformed external ears, defects of CNS, gross malformations of limbs (phocomelia, arthrogryposis), oligodactylies, polydactylies, syndactylies, defects of anterior body wall and some malformations of external genitalia.

Fetoscopy is indicated in syndromes which do not exhibit chromosomal or biochemical abnormalities inherited according to Mendelian laws, and which are characterized by constant anatomical malformations.

Diagnostic fetoscopy has been performed in our hospital by Zwinger in 47 cases; 20 of them were therapeutically aborted. Malformations were found in 18 cases. In two cases in which the fetuses were diagnosed fetoscopically as malformed, the diagnosis was not confirmed after abortion. Unwanted abortion occurred in four patients with healthy fetuses. Pre-term deliveries occurred in three cases, two pre-term newborns died. Twenty patients delivered healthy children at full-term. Leakage of amniotic fluid occurred in six of them. Using fetoscope we detected following defects: familial cleft-lip and palate (6 cases), Treacher Collins' syndrome (2 cases), anencephaly (previously diagnosed ultrasonically, 2 cases), ectrodactyly (1 case), Saldino-Noonan syndrome (1 case), Majewski syndrome (1 case), syndactyly type V (1 case), Roberts syndrome (1 case), arthrogryposis (1 case), Apert syndrome (1 case). Lip pit-cleft lip syndrome (1 case).

Sampling of fetal tissue under fetoscopic control. The needlescope, which has a rather limited visual field for a general fetoscopy is a good tool for visualization of chorionic veins in the placental plate. After a suitable vein is visualized, a heparinized needle is directed into the lumen of the vessel. By this method pure

fetal blood can be obtained and used for prenatal diagnosis of hemoglobin disorders (MacKenzie and Maclean, 1980).

Fetoscopy controlled intrauterine skin and muscle biopsies are possible and promising for diagnoses of disorders which are not detectable from amniotic fibroblasts. Fetoscope can be also used for the control of intrauterine transfusion.

3.3 Non-Invasive Methods in Prenatal Diagnosis

3.3.1 Ultrasonography

Using real-time ultrasonic instruments, the conceptus can be visualized during 5th or 6th gestational weeks, and its growth, position and localization of the placenta can be monitored (Joupilla and Piiroinen, 1975). The increase in the biparietal diameter is normally used for general growth evaluation (Queehan *et al.*, 1976). Estimations of fetal body and head volumes are performed to diagnose intrauterine growth retardation (Jordan and Clark, 1980).

The fetal heart rate and the beat of umbilical arteries can be also visualized ultrasonically. The following malformations can be diagnosed prenatally by ultrasound procedures: anencephaly at the beginning of the second trimester; myelomeningoceles, but diagnosis may be difficult (Campbell, 1977); hydrocephalus and microcephaly in the third trimester; iniencephaly; sacrococcygeal teratomas; multicystic or polycystic kidneys or hydronephrosis and omphaloceles visualized as masses in a characteristic location (Bartley et al., 1977). In some cases, the shape of external geintalia allows the diagnosis of sex. In the last trimester, the heart valves and cavities can be visualized and the diagnosis of gross congenital heart anomalies is possible (Patel and Goldberg, 1976).

3.3.2 Radiography

The use of direct radiography for diagnosis of skeletal defects is limited to the third trimester. During second trimester even gross anomalies such as achondroplasia are undetectable (Golbus and Hall, 1974). Prenatal radiographic diagnosis (after 30th gestational week) of anencephaly, hydrocephaly, microcephaly and of most of the gross skeletal anomalies has been reported. Contrast radiography with a water-soluble radio-contrast dyes has been used in the third trimester for visualization of absent fetal swallowing in cases of gastrointestinal atresia.

4 CONCLUSIONS

The elucidation of genetic, biochemical and physiological mechanisms involved in prenatal morphogenesis and embryonic function is a necessary prerequisite for

attempting the prediction of adverse effects of chemical agents on the exposed mother and fetus. To predict the effects of xenobiotic substances on prenatal development, the stage of pregnancy at which the fetus was exposed and the exposure concentration need to be known. Predictions based on animal embryotoxicity and teratogenicity are, however, of limited value.

For detection of inadequate fetal growth, chromosomal and metabolic diseases and external anatomical malformations, the following methods can be used: cytology of cells exfoliated into the amniotic fluid, direct biochemical tests of amniotic fluid, karyotyping of amniotic cells grown in tissue cultures, biochemical analysis of amniotic cells grown in tissue cultures, fetoscopy, analysis of fetal blood withdrawn under fetoscopical control, ultrasonography and radiography of the fetus.

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