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Human Placenta as a Biomarker of Environmental Toxins Exposure – Long-Term Morphochemical Monitoring

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1. Introduction

A healthy lifestyle, concerning the choice of diet and physical activity, has become increasingly important to human choice. However, even with the best of intentions and the best individual lifestyle choices, unfortunately we are not able to control all of the factors affecting our body, even in the simplest terms of chemical and physical pollutants of the ambient environment. Biomonitoring is the sphere of human interest regarding how a variety of environmental factors affect living organisms. In practice this means most frequently the elucidation of the adverse effects of environmental pollution, and looking for meaningful markers of such effects, which demonstrate not only the causal relationship with the agent but also a dose-proportionality of the causative agent. The efforts related to the monitoring make sense here, and can ultimately lead to the elimination of negative acting stimuli. In a large number of cases this is possible. Choice of human placenta for monitoring pollutants proven to be detrimental to human health has enabled us to lead real-time monitoring and long-term monitoring. With the exception of hair and placenta, ie transient organ which serves the needs of developing embryo and fetus, the other specimens used for real time monitoring require invasive procedures, or are obtained post-mortem.

The placenta binds two genetically distinct individuals, the mother and the fetus, and serves as an intermediary between maternal and fetal circulations. It is not merely a passive barrier between the maternal and fetal circulations, but has many physiological functions, including the exchange of respiratory gasses, metabolites, nutrients and waste products as well as the production of hormones and the metabolism of xenobiotics. The understanding of the mechanisms and rates of the maternal-fetal transport of chemicals at the placental level and the contribution of placental binding, storage and the metabolism of compounds is still being intensively studied, but offers many of scientific and practical benefits for vulnerable human populations.

The placenta is a unique sample requiring no invasive procedure, offers possibilities for real time monitoring, and serves for evaluating the pollutant burden exerted on the mother as well as on the embryo and fetus can provide powerful dosimeters for investigating reproductive effect.

1.1 Brief outline of placental biomonitoring

The possibility of using the placenta as an indicator of the chemical pollution and, accordingly, of the ecological value of human environments arose in the 1960s from the need to procure insight into the effect of fluorine compounds emitted by the Skawina Aluminium Smelter near Cracow on its employees and their families. At the same time the whole surroundings of Cracow used to be covered with a heavy metal dust fall and also oxides of carbon, sulphur, nitrogen and extremely many compounds of fluorine (Zamorska 1979, 1982/1983; Zamorska & Niweliński 1982/1083). The data elaborated in those years by Prof. Niweliński and Dr. Zamorska, pioneers of placental biomonitoring in Poland, have been handed over to the corresponding institution and finally contributed to the closure of the smelter in the 1980s. This was the first case of immobilization of the smelter as a result of social and scientific world protests in Poland. Many striking enzymatic and structural differences that were observed between the placentas of the Skawina women and those collected in mountainous, non-polluted regions of southern Poland, encouraging the use of the human placenta in the further monitoring of environmental pollution. This data supported by simultaneous analytical detection of trace elements and xenobiotics helped to control the excessive emission of industrial pollutants. Using the results of these first effective placento-ecological researches and their practical worth as a basis, the Department of Cytobiology and Histochemistry of Jagiellonian University opened in 1986 a new stage of researches for the monitoring of the environment pollution of a number of vulnerable South Poland regions, Upper Silesia and the Copper Basin, and as the comparative material placentas from the Carpathian Mountains - Bieszczady were taken. The choice of this Polish region was dictated not only by the distance from the industrial centers but also by the transport accessibility as well. Generally, Bieszczady is estimated as the least polluted region in South Poland and that is why Bieszczady placentas could be applied as the comparative material. During our 18 years of studies on the effect of environmental factors on the enzymatic and structural characteristics of the human placenta nearly 500 full term placentas from uncomplicated pregnancies of mostly non-smoking women, were collected in different regions of southern Poland and studied using histochemical, immunohistochemical and histological methods. In selected cases, a trace elements examination was applied. Prolonged studies of the effect of environmental chemical pollution on the biochemical and microanatomical organization of the human placenta show that this organ, when influenced by toxic factors, undergoes changes that are approximately proportional to the intensity of the pollution. The primary change consists of a histochemically detectable deterioration of cytochrome c oxidase in the villous syncytiotrophoblast when compared with the activity shown by placentas from sites of low pollution. This consequently results in a lack of energy supply and imminent placental insufficiency.

The researches of full-term human placentas as the substrate of monitoring lead to wide information both in the action for protection of the natural environment and in medical prophylaxis; especially in gynaecology and paediatrics.

1.2 Placental biomonitoring – examples of other challenges

Several other national scientific centers have developed programmes with a focus on ambient air pollution and the outcome of pregnancies and many these studies are of practical importance.

In the 1990s, an investigation “Teplice Program” was performed in the Czech Republic to evaluate the impact of air pollution on genetic material and reproductive outcomes (Binkova et al, 1999; Sram et al., 1996). Then the possible impact of air pollution on human reproductive quality was studied in the Pregnancy Outcome Project in the period 1994 – 1999. The effects of all monitored pollutants were studied in the background of a wide spectrum of potential confounding factors (parental biological, social and lifestyle parameters, ethnicity, seasonal factors, etc.) using logistic regression models. The most important findings of the Teplice Program were that air pollution may have an impact on adverse reproductive outcomes, DNA adducts and some genotypes are sensitive biomarkers of exposure and PAHs are an important source of the genotoxic and embryotoxic activities of organic mixtures associated with urban air particles. The Czech studies of the effect of air-pollution have demonstrated also a pronounced effect on intrauterine growth retardation (Dejmek et al., 2000), which is even more pronounced than the effect of active and passive smoking.

Bobak and Leon (Bobak & Leon, 1999) conducted an ecological study of low birth weight and the level of nitrous and sulfur oxides in many districts of the Czech Republic in 1986-1988. They concluded that only SO₂ was related to these adversary outcomes of gestation. Then, in the subsequent study (Bobak et al., 2001), they tested the hypothesis that air pollution is related to low birth weight on the data from a British cohort in 1946, and after researching and controlling a number of potential confounding variables ultimately found a strong association between birth weight and the air pollution index based on coal consumption (Sram et al., 2005). Ukrainian study was designed to analyze the effect of environmental oxidative stress on human placental monooxygenases, glutathione S-transferase (GST) activity and polycyclic aromatic hydrocarbon (PAH)-DNA adducts in human term placentas from radioactivity-contaminated and chemically-polluted areas of the Ukraine and Belarus, and to compare these biomarkers to the newborn’s general health status. Environmental oxidative stress was related to an increase in anemia, threatened abortions, toxemia, fetal hypoxia, spontaneous abortions and fetal hypotrophy (Obolenskaya et.al., 2010). Further Polish data of study on children (Kubiak et al., 1993) confirms the cytogenic damages from environmental exposures. Although a detailed review of the studies on the effects of pollution on reproductive outcome is beyond the volume of this scope, this gives a view of interest concerning the problem of children’s vulnerability in the first period of their development and generally reproductive effect to the state of the environment.

2. Morphoenzymatic monitoring

2.1 Area of interest – Ecological background

The ecological hazard area is the territory where, as a result of intense human activity, the degradation of the components of the natural environment lead to the infringement of the ecological balance. Degradation is the result of repeated and prolonged doses of water and air pollution exceeding the considered safe limit.

2.1.1 Upper Silesia

The Polish Ministry Cabinet decided in 1983r that the Upper Silesia region was recognized as an ecologically impendent/hazard one. The extremely dramatic state of the

environment in this region was the consequence of a greater emission of gases and dust pollutions coming from the developing industry in the main coal basin in Poland. The mining of not only coal but also other raw materials (zinc and lead ores, etc) and their processing caused irreversible changes on the natural environment. The industrial refuses of Upper Silesia amounted to one third of the refuses in the entire country and, additionally, 1/6 of the total municipal waste amount produced in the country. A significant percentage of untreated toxic wastewater used to enter into rivers. Improvement of the ecological environment both in the air's sanitary state and in the quality of surface flowing waters could be observed after two decades of continuous monitoring (for details see fig. 10), although the state of health of the Upper Silesia population left much to be desired. Very importantly, it was revealed that a high number of mortality and morbidity among children was simultaneous with the high coefficient of prematurity and supermortality of newborn children (Burton et al., 1989).

2.1.2 Polish Copper Basin

The Polish Copper Basin is the one of main industry centers in Poland and the one of the biggest centers of copper in the world. The copper industry development, not always well organized considering the ecological responsibilities, generated a huge amount of gas impurities as well as metallurgical dust. The emission of pollution was so high in this region that the Copper Basin was claimed to be the most ecologically hazardous region in the 1980s. It was not until in the '90s that a significant decreasing of the emission dimension (both gases and dust) to the atmosphere was observed. Analysis of data from 1980-1995 (Hławiczka, 1998) proved that the main components of emitted compounds were the following metals: copper, lead, arsenic and zinc. In spite of the diminishing emission level of the heavy metals, the purity of the atmosphere, plants and soil were still disappointing. The effect of pollution immediately was transposed into human health, especially children. According to the researches of the Foundation for Children from the Copper Basin, it turned out that the number of children with a higher level of Pb concentration in their blood, which is more than 10 µg/dl, was in 1996 yet 13.6% (Strugała-Stawik & Stawik, 1999). Moreover, amongst the Copper Basin population, diseases of the respiratory system, blood system, stomach-intestinal troubles, liver dysfunctions evidenced human intoxication in SO₂ and Cu were more commonly observed.

2.2 Samples collection and methods

The examined material consisted of 493 full term human placentas from non-complicated pregnancies. Among them, 197 placentas were collected during the years 1985-2002 in Upper Silesia, 69 - during the years 1995-1998 in the Copper Basin and 227 during the period of 1985-2002 in the low polluted South-Eastern Carpathian Mountains. The latter placentas served as control material. All the mothers (placenta donors) were healthy and free from alcoholic and smoking habits and throughout their gestation time they did not change their dwelling places. All the neonates were healthy. A detailed inquiry was made with each of the women who supplied the placentas. The inquiry pertained to the woman's age, her general health state, genetic constitution and diseases she had in the past. Similarly, the corresponding data and sex of each neonate was noted.

2.2.1 Tissue preparation

Immediately after parturition the cord was clamped. The umbilical cord, the blood coagula and membranes were then carefully trimmed and each placenta weighed and the volume measured by fluid displacement. Approximately 12 full thickness cores of placental tissue (fetal subchorial to maternal parabasal surface) were generated, at an equal rate, from the area close to the umbilical cord insertion, from the peripheral region and from the interspaces between them in a random manner. A part of the tissue cores was processed to paraffin using routine laboratory techniques. For histochemical purposes the blocks were instantly frozen using dry ice and cut to meet the requirement for vertical full depth slices, yet haphazardly of the x,y - cutting plane to ensure isotropy to the estimation surface cross-section and numerical density of placental structures. For investigation into the concentration of trace elements, full-thickness tissue samples were uniformly excised from the central and pericentral areas of each placentas and from fetal membranes using sterile conditions and materials. All specimens were kept frozen until further use.

2.2.2 Histochemistry

The frozen placental portions were sectioned at 6 μm in a cryostat and processed for the detection of the following oxidative enzymes: cytochrome c oxidase (CCO - EC 1.9.3.1) by the modified Burstone method (Stoward & Pearse, 1991), NADH dehydrogenase (NADD - EC 1.6.99.3) by the method of Pearse (Pearse, 1972) and glucose-6-phosphate dehydrogenase (G6PD - EC 1.1.1.49) according to Van Noorden and Vogels (Stoward & Pearse, 1991). We detected also lactate dehydrogenase (LDH - EC 1.1.1.27) and LDH-1 and LDH-5 isoenzymes by the standard method of Mac Millan (Mac Millan, 1967). The histochemical reaction applied for NADH made it possible to simultaneously study the morphological character of the structures of interest.

2.2.3 Trace element study

The concentration of trace elements in the placental and fetal membrane samples were studied using the total reflection X-ray fluorescence (TRXRF) method. This research was conducted in the Institute of Physics, Świętokrzyska Academy of Kielce, Poland. The TXRF method is known to be well suited to study trace elements in biomedical samples. For details of this method see ref. (Majewska et al., 1999). Moreover, the semiquantitative evaluation of the contents of mineral deposits in placental tissue, using an arbitrary scale of five degrees (from 0 to 4 in ascending gradation order), was performed.

2.2.4 Morphometric analysis

Morphological analysis was undertaken at a light microscopy level aided by the computer software Multiscann 6.08 and using histochemical placental tissue sections processed for the demonstration of the NADD activity. This was because the histochemical procedure did not produce the shrinking effect in the internal structures which generally, otherwise, appear in tissues treated with histological fixatives. All tissue sections were coded and subsequently examined blindly with the environmental information. An average of 6 randomly selected serial sections from central and pericentral placental areas were analyzed. Test point counting was used to estimate the placental intervillous space density. Diameters of the villi were estimated by direct measurement. The total counting number of villi and this number

then divided into classes with increasing dimensional was referred to as the 1mm^2 placental cross-section area yielding numerical density value. A villi classification scheme describes Tab. 2 and its relation to this as described by Mayhew (Mayhew, TM. 2002, Benirschke, K. 2006), classes 1 and 2 both refer to terminal villi, class 3 refers to the largest terminal villi and to intermediate mature villi, class 4 embraces large immature intermediate villi and class 5 - stem villi. To ensure the uniformity of measurement approximately 60 systematic random microscopic fields of view were analyzed per placenta.

2.2.5 Statistical analysis

Statistical analysis was accomplished using the t-Student test and its most sensitive nonparametric analogue the Mann-Whitney U test to show the differences between the two groups. To compare with each other more than two groups, the Kruskal-Wallis test and Dunn's post-hoc test were applied. The median test was used to assess the significance of the differences between the data found in the rank order. To assess correlation values between the selected parameters, the Pearson or Spearman correlation were used. The p values below 0.05 were considered as significant.

2.3 Some aspects of the development and morphology of the placenta in the context of monitoring

Prior to entering into details of the complicated effect of the xenobiotic factor on the morphological and histoenzymatic characters of the human placenta it seems reasonable to have a general view of the structure and function of this materno-fetal organ, at least in respect of its use in biomonitoring. A well-developed placenta consists of a chorionic plate, which is of embryonic descent and of a basal plate whose essential layer is the decidua, a derivative of the endometrium. Between these two plates there is a voluminous intervillous space.

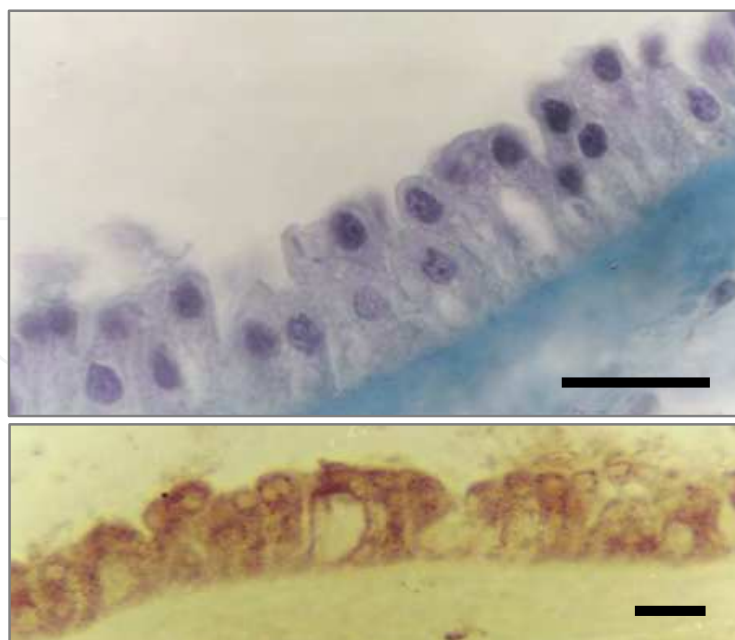


Fig. 1. Cryostat cross-section through fetal membranes from the control material. Histological trichrome Masson stain (upper panel) and histochemical reaction for cytochrome c oxidase. Bar: 20 μm .

The upper surface of the chorionic plate is covered by the rather uniform amniotic epithelium (Bilic et al., 2004), which is in contact with amniotic fluid directly, whereas the lower surface of that plate presents an abundance of villous trees which are ramified and richly vascularized outgrowths of the plate. Each villous tree consists of a stem villus (over 300 μm in diameter) whose cells by gradual proliferation progressively arborize and form branches in descending order of length and thickness. The thinnest and shortest branches of every villous tree are the placental terminal villi, which are structures of the uppermost functional importance for placental efficiency.



Fig. 2. Paraffin cross-section through crucial for transplacental exchange terminal villous (methyl blue stain). It comprises of outer trophoblastic epithelium which surrounds mesodermal stroma containing fetoplacental capillaries. The epithelium is composed of proliferative cytotrophoblast cells and a superficial terminally-differentiating syncytiotrophoblast. Bar: 15 μm .

It is through the walls of these villi that the great majority of the exchange of materials between the fetal blood in the villous blood vessels and the maternal blood flowing through the intervillous space occurs. The surface of the villous trees is coated by a double layer of trophoblast, under which there is a well vascularized mesenchymal core. The superficial layer of the trophoblast is a syncytium; i.e. the syncytiotrophoblast that abuts on the underlying layer of the cytotrophoblast. The cytotrophoblast is capable of mitotic divisions that do not occur in the syncytiotrophoblast. Throughout the gestation period, the cytotrophoblast replenishes and restores the syncytiotrophoblastic layer which with the progress of time gradually becomes used up and destroyed by forming the syncytial knots. The whole trophoblast coat also gradually expands and finally completely covers the entire wall of the intervillous space. The cytotrophoblastic cells enter and dilate the spiral maternal arteries in the basal plate and thus augment the maternal blood supply to the fetoplacental unit. This blood is rich in indispensable oxygen and nutrients obtained from the mother.

The previously emphasized great role of the terminal villi is made possible by the many activities of the syncytiotrophoblast which, above all, is responsible for the adequate oxygen supply to the fetus. Amino acids, polypeptides and proteins including maternal antibodies protecting the fetus against dangerous antigens and infections, plus many vitamins, pass from the maternal to the fetal blood by active transport accomplished by the syncytiotrophoblast.

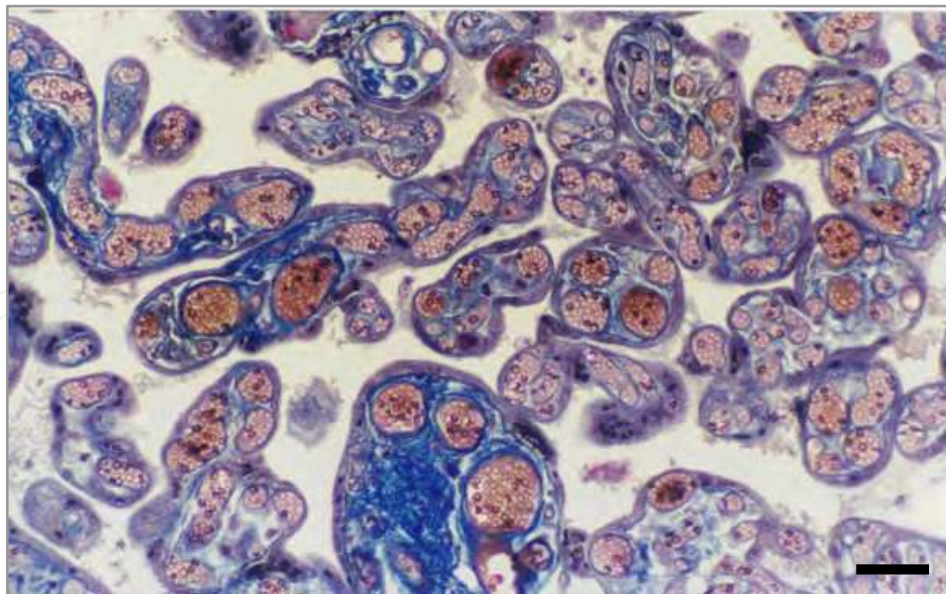


Fig. 3. Paraffin cross-section of a full term control placenta. Histological trichrome Masson stain. Bar: 30 μ m.

Active transport as the process occurring against chemical gradients requires expenditure of placental energy accumulated in ATP, which is produced by the trophoblastic mitochondria only in the presence of oxygen. Other important functions of the syncytiotrophoblast are synthesis, metabolism and secretion of hormones including human chorionic gonadotropin, human chorionic somatotropin, steroid hormones progesterone and oestrogens hypothalamic hormones, e.g. the corticotropin releasing hormone (factor) CRH. Removal of fetal waste products also requires cooperation of the syncytiotrophoblast. All these facts demonstrate the absolutely essential role of the villous trophoblast in the placental and fetal physiology (Benirshke et al., 2006).

2.4 Maternal and neonatal findings

By contrast to the maternal characteristic, e.g. mothers age and gestational age, the neonatal findings (tab. 1), were significantly different between groups. A newborn child's birth weight and length from the polluted environment show significantly lower values. More detailed studies revealed that differences in the weight of the neonatal of different sexes were much more pronounced in the polluted area (3189 g for female newborn and 3369 g for male newborn; $p < 0,05$) than in the control (respectively 3443 g and 3490 g). Also, the placental weight, although not its volume, was significantly lower in the contaminated environment. Measurements of weight and length of newborns from Upper Silesia showed a strong Pearson correlation in respect of one another; i.e. newborns weight terms of placental weight ($r = 0.6738$, $p < 0.001$), newborn weight terms of its length ($r = 0.7479$, $p < 0.001$) and placental weight terms of the length of the newborn ($r = 0.6738$, $p < 0.001$).

These findings strongly suggest that ambient air pollution, industrial emissions accurately and/or other factors associated with residence near a exposed region during the pregnancy, affect fetal growth. A great number of other studies of air pollution and birth outcomes also clearly evidenced that pregnancies are susceptible to the adverse effects of air pollution and the evidences are sufficient to infer causal relationship between air pollution and birth

	Upper Silesia (n = 197)	Control (n = 227)	p value
Mothers age (years)	26.2 ± 1.1 (median - 25)	27.1 ± 1.1 (median - 26)	NS
Gestational age (weeks)	39.6 ± 0.1 (38-42)	39.5 ± 0.1 (38-42)	NS
Newborn`s birth weight (g)	3262 ± 35 (2100-4420)	3571 ± 33 (2950-4700)	p = 0,003
Newborn`s length (cm)	54.1 ± 0.2 (48-62)	55.3 ± 0.2 (48-64)	p < 0.001
Placental weight (g)	461 ± 8.3 (220-650)	497.6 ± 7.9 (260-780)	p = 0.015
Placental volume (cm ³)	465 ± 15.4 (230-680)	486 ± 9.6 (300-800)	NS

Table 1. The clinical characteristics of studied groups. Data are presented as mean ± SEM.; (range or median); t-Student test for comparison of two studied groups was used. NS, not significant.

weight (Bobak & Leon 1999b; Bobak et al.; 2001; Rich et. al 2009) in addition to other adverse reproductive outcomes, including premature births, intrauterine growth retardation and ultimately infant deaths, whose range does not overlap with our study.

2.5 Morphological changes in placentas from polluted areas

The data presented above evidenced that in conditions of severe ambient exposure decrease not only infant’s weight but also impair the development of the placenta. Therefore it was necessary to try to explain how it changes the internal placental structure which is closely affiliated with their functions. It was therefore necessary to examine how this changed their internal morphology, which after all is closely affiliated with their functions. Our presented data provides clear evidence of rebuilding the structure of villous trees. The numerical density of the villi measured in placentas’ cross-sections strongly increases (tab. 2). This increase was primarily generated by the small dimensional terminal villi. Our working distinction of terminal villi to two separate classes enabled us to realize that there was an increase in the density of small dimensional terminal villi (class 1), while the terminal villi of typical dimensions (class 2) remained at the same level of density than in the control placentas. It should be underlined here that only fully histological qualified terminal villi were included to class 1, avoiding syncytial knots and trophoblast sprouts, and even neck regions with unclear characteristics. Also, other types of placental villi, i.e. intermediate mature villi and immature ones, and mesenchymal villi, included in other classes, did not show the revised density. Therefore we claim that because of the overproduction of small dimensional terminal villi, the differences in the percentage of individual villous class terms of the other ones became statistically significant. Furthermore, we consider the phenomenon of overproduction of terminal villi with small dimensions to be an important indicator of the impact of a polluted ambient environment on human placental developmental processes. It can be assumed that this change is caused by adaptive processes in the direction of increasing the surface area of the gas transfer and transport of nutrients.

The structure of the villous tree develops on the basis of proliferative and angiogenic processes, and the functions of angiogenic factors (Mayhew, TM. 2002). Intraplacental oxygen status has effect on the control of the angiogenic growth factor production, and in consequence villous differentiation. A lot of the phenomena regarding changes in the placental morphology and the adaptive processes are explained on the ground of materno-placento-fetal hypoxia (Mayhew et al., 2004). Hypoxia has been sub-classified into pre-placental, uteroplacental and post-placental in source (Bush et al., 2000). Preplacental

hypoxia means that maternal blood is hypoxaemic when it enters the intervillous space. In uteroplacental hypoxia, normoxic maternal blood flows into intervillous space, but disturbances appear locally in the blood flow and the placenta suffers from a heterogenous oxygen supply. In postplacental hypoxia, fetal oxygen extraction from the placenta is diminished (Kingdom & Kaufmann, 1997). Oxygen-dependent growth factors are essential in regulating angiogenesis and switch this process into a branching or non-branching path and promote or inhibit terminal villous development (Egbor et al., 2006; Kaufmann et al., 2004). There is now a reasonable body of evidences that the placental villous trees with adverse stimuli can adapt to the attendance in order to maintain an effective level of transport for the gasses and nutrients. In pregnancies under the impact of fetal hypoxic stress (high altitude or anemia), changes in transport efficiency are variable and may reflect the differences in sources of hypoxia (Mayhew et al., 2004). The comparisons show that the pattern of changes in pregnancies exposed to all industrial pollution is not fully consistent with any other patterns but are close to that of uteroplacental hypoxia with reduced intraplacental oxygenation. The main point of a coherent with a uteroplacental pattern of hypoxia is that it causes predominantly branching angiogenesis following by terminal villous overproduction. Formation of new villi is not the only consequence, as branching angiogenesis leads to reduced vascular impedance in the fetal blood vessel. Simultaneously, a denser villous arrangement takes up intervillous space (tab. 2, fig. 2) which restricts, on the other hand, the maternal blood supply and the physical abilities to diffusion and nutrient transport. In other words the incorrect switching towards the hypermature differentiation results that despite its highly differentiated villi, placenta may not provide enough exchange level.

	Diameter (in relation to classes of villi)	Upper Silesia (n = 197)	Control (n = 227)	p value
Density of all villi classes (per 1 mm ²)		142.92 ± 3.4	125.27 ± 3.1	p < 0.001
Class 1 of villi (per 1 mm ²)	below 40.50 µm	32.81 ± 2.6	13.77 ± 2.5	p < 0.001
Class 1 of villi (%)		21.81 ± 1.6	10.71 ± 1.3	p < 0.001
Class 2 of villi (per 1 mm ²)	40.51 µm – 59.50 µm	69.45 ± 2.9	68.76 ± 2.4	NS
Class 2 of villi (%)		48.74 ± 1.6	54.01 ± 1.6	p = 0.003
Class 3 of villi (per 1 mm ²)	59.51 µm – 89.50 µm	30.62 ± 1.1	31.64 ± 1.3	NS
Class 3 of villi (%)		22.07 ± 0.8	25.39 ± 0.7	p < 0.001
Class 4 of villi (per 1 mm ²)	89.51 µm – 119.50 µm	6.57 ± 0.5	7.62 ± 0.4	NS
Class 4 of villi (%)		4.81 ± 0.4	6.28 ± 0.3	p = 0.002
Class 5 of villi (per 1 mm ²)	119.51 µm – 159.50 µm	2.18 ± 0.2	2.63 ± 0.2	NS
Class 5 of villi (%)		1.67 ± 0.1	2.18 ± 0.1	p = 0.015
Intervillous space (%)		20.46 ± 0.5	25.19 ± 0.5	p < 0.001

Table 2. The numerical density of all villi classes and percentage of intervillous space. Data are presented as mean ± SEM.; t-Student test for comparison of two studied groups was used. NS, not significant.

In the proper developmental processes through the third trimester, the mesenchymal villi become preferentially transformed into mature intermediate villi. The surface of the latter, as we said, is protruded by elongating and looping fetal capillaries resulting in protrusion of

highly specialized of materno-fetal exchange terminal villi. There is no longer transformation of mesenchymal villi into mature intermediate villi - the remaining population of immature intermediate villi differentiate into stem villi. Thus their number steeply decreases toward the term and, together with this the base for the formation of new mesenchymal villi sprouts, and the growth capacity of the villous trees gradually slows (Benirshke et al., 2006, Castellucci et al., 2000). Meanwhile, immature intermediate villi are constantly strongly represented in the exposed placentas (tab. 2).

2.6 Histoenzymatic changes in placentas from polluted areas

The morphological adaptations previously described are affiliated by variations in the normal metabolic paths in the placenta. We investigated the following oxidative enzymes: cytochrome c oxidase, NADH dehydrogenase and glucose-6-phosphate dehydrogenase. We detected also lactate dehydrogenase LDH-1 and LDH-5 isoenzymes in villous trophoblast and fetal membranes.

Cytochrome c oxidase is the ultimate enzyme of the mitochondrial electron transport chain located in the mitochondrial membrane and its activity is required for the cell energy supply. In placental trophoblast nutrient transport, protein synthesis and other processes need a continuous supply of energy and in this perspective cytochrome c oxidase is the key enzyme in the regulation of placental functions. Placental insufficiency to energy delivery restricts fetal growth. We observed a significant decrease in the histochemically detectable activity of mitochondrial cytochrome c oxidase in the trophoblast of exposed placentas. A detailed discussion of the origin of mitochondrial insufficiency or causal link between exposure and hypoxia is beyond our present scope. Decreased cytochrome c oxidase activity or the decrease of cytochrome c oxidase positive mitochondria may be a result from placental ischemia which is an inherent occurrence in pre-eclampsia. While the mechanism of the reduced cytochrome c oxidase activity observed here is unknown, such a reduction may impair the production of placental energy, resulting in placental insufficiency in patients and the deterioration of the newborn. Damage to the cytochrome c oxidase activity, which is responsible for the intensity of aerobic oxidation and thus for the intensity of metabolism, is causally related to compensatory efforts that the feto-placental unit undertakes to save its function and homeostasis (tab. 3, fot. 4, 5).

The mean activity of enzymes (optical degrees)				
Enzymes		Upper Silesia (n = 197)	Control (n = 227)	p value
Cytochrome c oxidase	trophoblast of villi	79.9 ±2.7	123.8 ± 1.9	p < 0.001
	amniotic epithelium	66.2 ± 4.7	120.2 ± 3.7	p < 0.001
NADH dehydrogenase	trophoblast of villi	155.3 ± 2.2	167.2 ± 1.1	p < 0.001
	amniotic epithelium	171.8 ± 4.6	184.4 ± 3.6	p = 0.009
Glucose-6-phosphate dehydrogenase	trophoblast of villi	106.1 ± 3.9	127.6 ± 1.8	p < 0.001
	amniotic epithelium	112.8 ± 7.5	149.6 ± 5.4	p < 0.001
Lactate dehydrogenase LDH-1	trophoblast of villi	56.3 ± 4.6	80.5 ± 2.9	p < 0.001
	amniotic epithelium	35.2 ± 5.5	70.8 ± 4.3	p < 0.001
Lactate dehydrogenase LDH-5	trophoblast of villi	35.4 ± 3.5	26.2 ± 1.7	p = 0.031
	amniotic epithelium	133.8 ± 8.7	106.5 ± 6.7	p = 0.002

Table 3. The mean activity of intracellular enzymes in optical degrees. Data are presented as mean ± SEM. t-Student test for comparison of two studied groups was used.

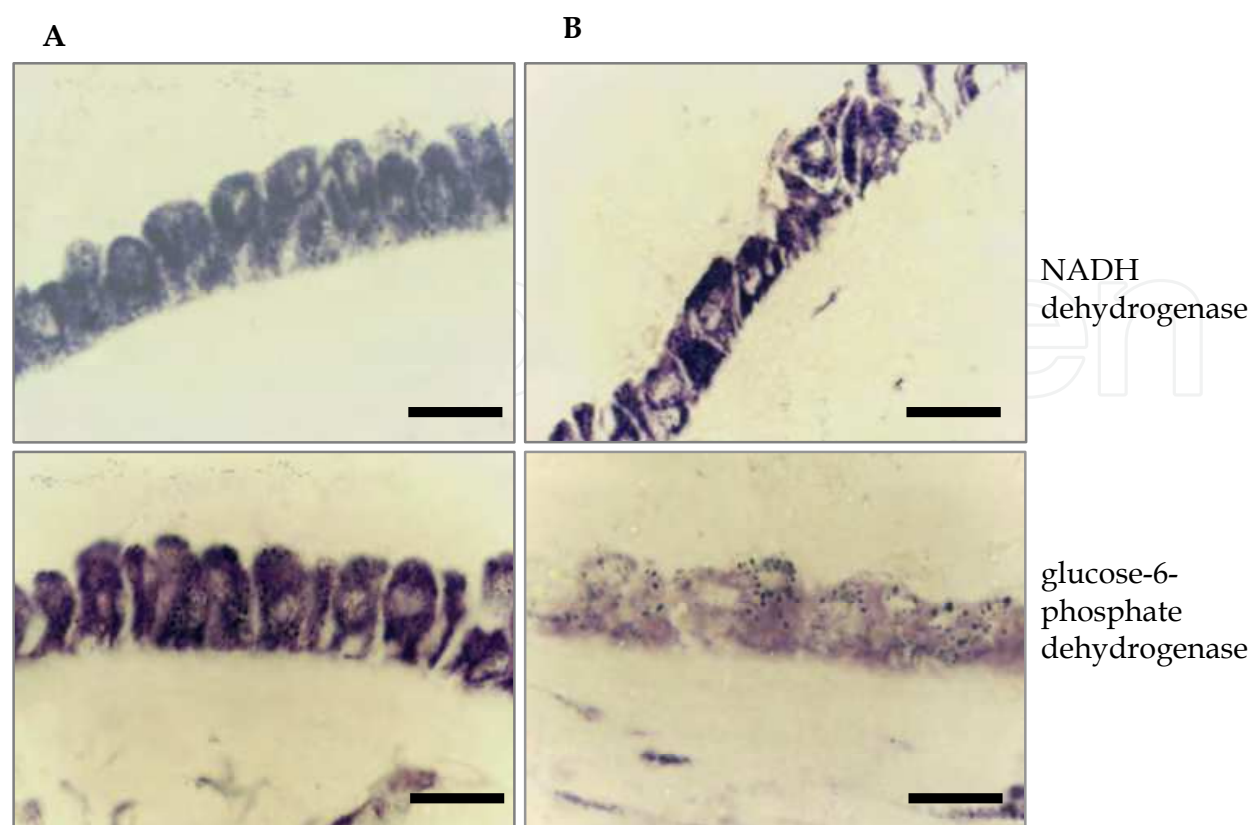


Fig. 4. Cryostat cross-section through amniotic membranes from control (column A) and polluted (column B) environments. Histochemical reactions for intracellular enzymes. Bar: 20 μ m.

Similar changes, although with not as dramatic a reduction in activity, were observed in the case of NADD, which is an enzyme located in the inner mitochondrial membrane and catalyzes the transfer of electrons from the NADH to coenzyme Q, and is claimed to be the "entry enzyme" of oxidative phosphorylation in the mitochondria (tab. 3, fot. 4, 5).

Glucose-6-phosphate dehydrogenase also showed a decreased activity in the villous trophoblast of exposed placentas (tab. 3, fot. 4, 5). G6PDH catalyses the first step of the pentose phosphate pathway and then generates the reducing form of NADPH and produces pentose phosphates necessary for nucleotide biosynthesis and ultimately serves as the path of entry for pentoses to the glycolytic pathway. In the placenta, which is the transport gate for substances to the fetal bloodstream, and at the same time a barrier for many other ones, the reducing power of NADPH cannot be overestimated, due to its relationship with glutathione disulfide reductase. Glutathione and its disulfide protect the cell against the undesirable effect of noxious chemicals, which might be transferred from maternal blood (Myllynen et al., 2005). Next to the generation of reducing equivalents, the pentose phosphate pathway contributes to the production of ribose-5-phosphate used in the synthesis of nucleotides and nucleic acids, and erythrose-4-phosphate, used in the synthesis of aromatic amino acids. In valid metabolism, the increased utilization of NADPH would trigger G6PDH activity. Meanwhile, in the case of exposed placentas we observed a diminishing G6PDH activity in villous trophoblast and fetal membranes, which in turn, in accordance with prescribed metabolic directions impacts on fetal growth.

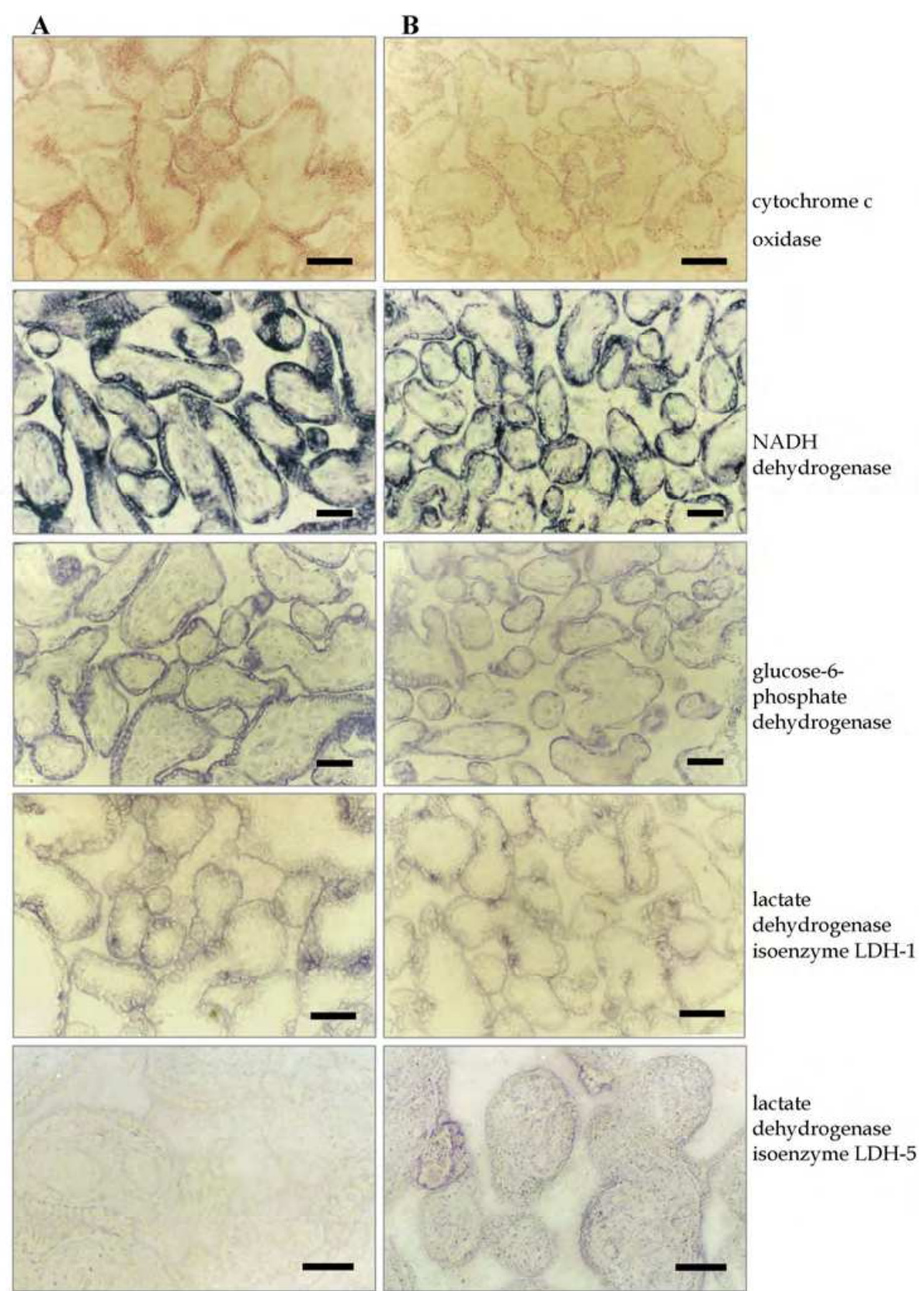


Fig. 5. Cryostat cross-section through full term placentas from control (column A) and polluted (column B) environments. Histochemical reactions for intracellular aerobic and anaerobic enzymes. Bar: 25 μ m.

A very expressively presented the distribution of isoenzymes of lactate dehydrogenase. LDH is responsible for pyruvate conversion to lactate through the glycolysis and is an important enzyme in the placenta because glucose is its main metabolic substrate and glycolysis is the major metabolic pathway. LDH-5 isoenzyme efficiently catalyzes pyruvate to lactate regenerating co-factor NAD⁺ under anaerobic conditions (Tsoi et al., 2000). This study is the first, to our knowledge, to demonstrate there is a differential expression of LDH isozymes in placental tissue when exposed to noxious ambient air. We found that exposed to ambient pollution placenta predominantly express the LDH-5 isoenzyme in villous trophoblast. Selective expression of LDH isoenzymes in syncytiotrophoblasts and villous matrix cells in the villi may reflect functional differences in LDH isoenzyme activity related to energy. As the LDH-5 isoenzyme has a higher affinity binding to pyruvate and NADH than the LDH-1 isoenzyme, it is more directly responsible for the production of lactate and NAD⁺. The high level of LDH-5 expression in a polluted environment, proves that villous trophoblast may be more capable of utilizing pyruvate as an energy source through anaerobic glycolysis under a hypoxic environment. The LDH-5 gene is a well-characterized hypoxia-inducible gene among many other glycolytic enzyme genes (Semenza et al., 1994). Given the upregulation of the LDH-5 gene expression shown in this study, the evidence supports the role for hypoxia within the metabolic/morphological changes in the placenta. In contrast to LDH-5, the LDH-1 isoenzyme is expressed constitutively and is the predominant enzyme in the cytotrophoblasts throughout the normal development of the placenta, as also evident in our study (fig. 4, tab. 3). On the other hand, the ascending lactate production has an effect on the VEGF level increase (Kay et al., 2007), which contributes in turn to the branching angiogenesis associated with hypoxia and the consequently observed generation of a new population of terminal villi (discussed above).

2.7 Placental indicators based on the long-time monitoring

The placentas of donors dwelling in polluted regions showed an advanced degradation of the activity of cytochrome c oxidase (fig. 6) and other oxidative enzymes of utter importance to the sustaining of life processes, particularly for the generation of energy indispensable for the active transport of substances exchanged between the mother and her fetus to occur. The substantial changes included an increase in the number of the terminal placental villi and thus an enlargement production of the inner exchange surface of the pollution affected placentas at the cost of the dimension of the placental intervillous space (fig. 9) and lessened supply of nutrients to the fetus. The children born with placentas showing histochemical changes were remarkable for their poor birth weight. Both the placentas and children in the low polluted region presented a full contrast with their counterparts from Upper Silesia (fig. 7).

The considerable amount of reciprocal correlations in turn of various features identified in exposed placentas give the strong confirmation of hypothesis on morphological and metabolic modifications in follow of intraplacental hypoxia (tab.4, fig. 6-9). The particular attention is paid to placental markers based on the strong correlations found in exposed placentas between the oxygen-dependent and this operated in lack of proper oxygenation metabolic and morphologic features: inverse correlations between the CCO activity vs the mineral deposit occupancy ($r = -0.2468$, $p = 0.012$), the numerical density of small dimensional terminal villi (tab. 4, fig. 9), and the LDH-5 activity (tab. 4, fig. 9), and in turn positive correlation between the LDH-5 activity vs the numerical density of small

List of Pearson correlations for Upper Silesia samples		
CCO vs LDH-1	r = 0.5550	p < 0.001
CCO vs NADD	r = 0.3703	p < 0.001
CCO vs G6PD	r = 0.2752	p = 0.038
CCO vs LDH-5	r = - 0.2715	p = 0.034
NADD vs LDH-1	r = 0.4666	p < 0.001
NADD vs G6PD	r = 0.3353	p = 0.011
CCO vs total number of villi (per mm ²)	r = - 0.2633	p = 0.013
CCO vs class 1 (per mm ²)	r = - 0.5248	p < 0.001
CCO vs class 1 (%)	r = - 0.5378	p < 0.001
CCO vs class 2 (%)	r = 0.4033	p < 0.001
CCO vs IVS	r = 0.3669	p < 0.001
LDH-1 vs total number of villi (per mm ²)	r = - 0.3106	p = 0.028
LDH-1 vs class 1 (per mm ²)	r = - 0.4955	p < 0.001
LDH-1 vs class 1 (%)	r = - 0.5284	p < 0.001
LDH-1 vs class 2 (%)	r = 0.4379	p = 0.002
NADD vs class 1 (%)	r = - 0.2269	p = 0.037
NADD vs class 2 (%)	r = 0.2733	p = 0.012
G6PD vs class 1 (per mm ²)	r = - 0.3267	p = 0.027
G6PD vs class 1 (%)	r = - 0.4122	p = 0.004
LDH-5 vs class 1 (%)	r = 0.2950	p = 0.048

Table 4. List of Pearson correlations for findings from Upper Silesia samples.

dimensional terminal villi (tab. 4, fig. 9). On the other hand, in the control placentas the LDH-5 activity correlated negatively with small dimensional terminal villi. It is worth noting that the introduction of improved industrial technology and of unleaded fuel over the last 20 years (fig. 10) has led to an increase in the levels of cytochrome c oxidase and LDH-1 activity in placentas from Upper Silesia regions .

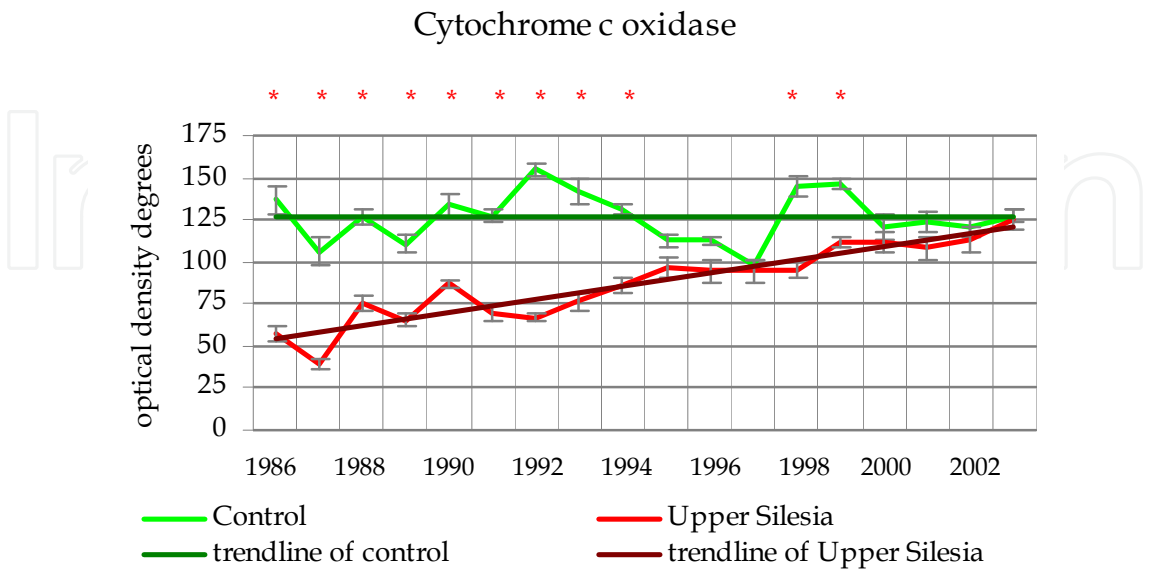


Fig. 6. The mean annual cytochrome c oxidase activity of Upper Silesia and control placentas in period 1986 – 2003.

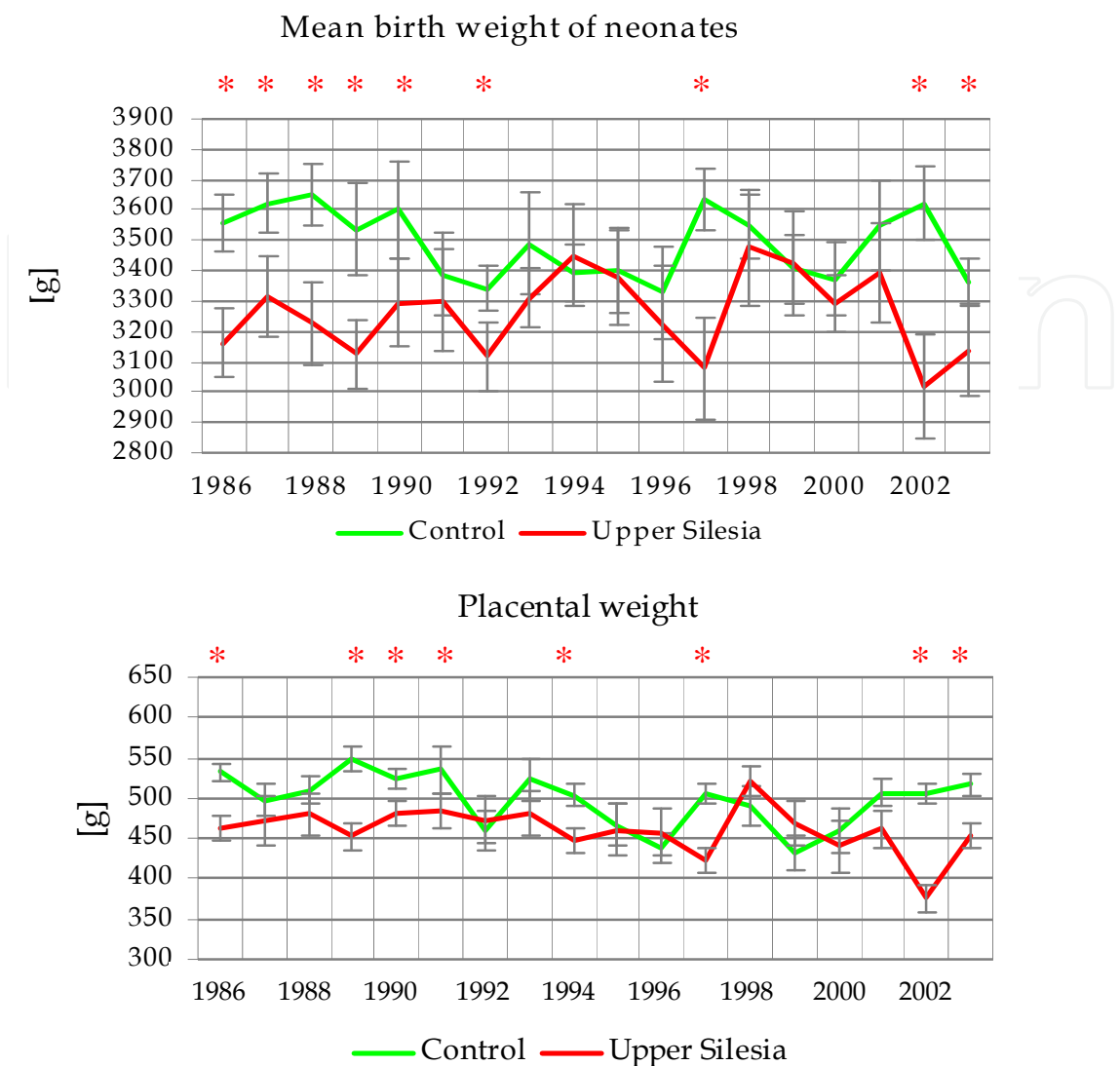


Fig. 7. The mean annual birth weight of neonates and placental weight in period 1986 - 2003. Comparison between control and Upper Silesia regions.

3. Essential and toxic elements in placentas and fetal membranes from Polish Copper Basin and their relationship to neonates birth weight and sex

The results of numerous studies conducted in recent years confirm the negative effect of the environmental contamination with heavy metals on the health and general biological condition of the population living in the areas threatened ecologically. The harmful effects of the environmental exposures depends on the type of contaminants, their concentrations and duration of exposure, as well as from the so-called ontogenic susceptibility.

Pregnant women, especially from industrial areas, are exposed to a wide variety of environmental toxins. During pregnancy, all of them are transferred from mother to fetus across the placenta. The placenta functions as a selective barrier and often bioaccumulates specific environmental toxic substances, including metals (Kantola et al., 2000). They may interfere with placental functions, causing placental damage and a change in the transport of essential trace metals to the fetus.

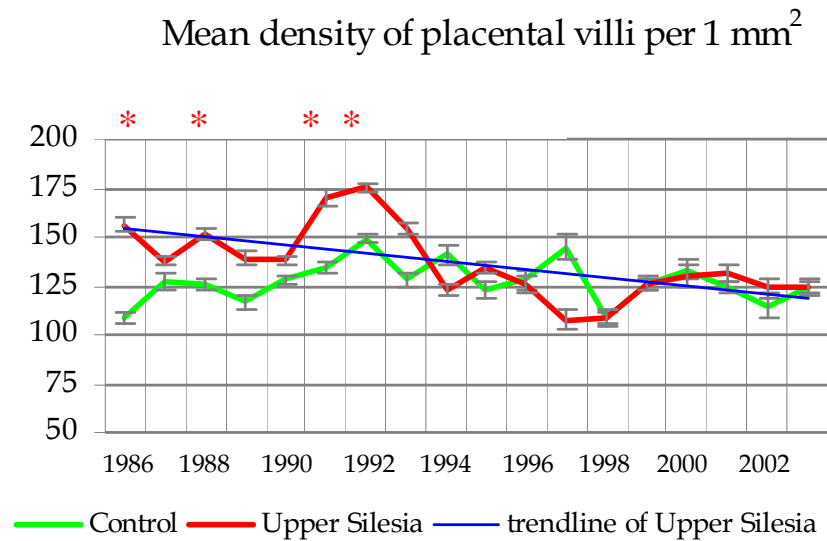


Fig. 8. The mean density of placental villi per 1 mm² of cross-section of Upper Silesia and control placentas in period 1986 - 2003.

Environmental exposure to heavy metals such as mercury, cadmium and lead is a serious growing problem throughout the world. These metals serve no biological function and their presence in tissues reflects contact of the organism with its environment. Their toxicity is frequently the result of long term, low level exposure to pollutants. Metal toxins have the ability to impair not just a single cell or tissue, but many of the body's systems that are responsible for our behavior, mental health, and proper physiological functioning. Essential minerals, such as zinc, copper, iron, selenium, chromium, manganese and calcium, may play an important role in decreasing the risk of the toxicity of heavy metals. On the other hand, a deficiency of these essential elements increases the toxicity of heavy metals (Sorell & Graziano, 1990; Kantola et al., 2000). Knowledge of the mineral interactions is essential in order to counteract the harmful effects of environmental exposure.

In the present study, we investigated some of the essential elements such as zinc, copper, chromium, manganese, calcium and toxic metals such as lead and mercury concentrations as well as mineral deposit contents in the placentas and fetal membranes of male and female neonates from the Polish Copper Basin.

3.1 The clinical characteristics of the studied groups

The examined material consisted of 88 placentas and 68 fetal membranes collected in the years 1995-1988. Of these, 50 placentas and 32 fetal membranes were collected in the Copper Basin and 38 placentas and 36 fetal membranes in the Carpathian Mountains – the control region. To avoid the seasonal variations in metal content, all of the samples were obtained during the autumn. The clinical characteristics of the studied groups are detailed in table 5.

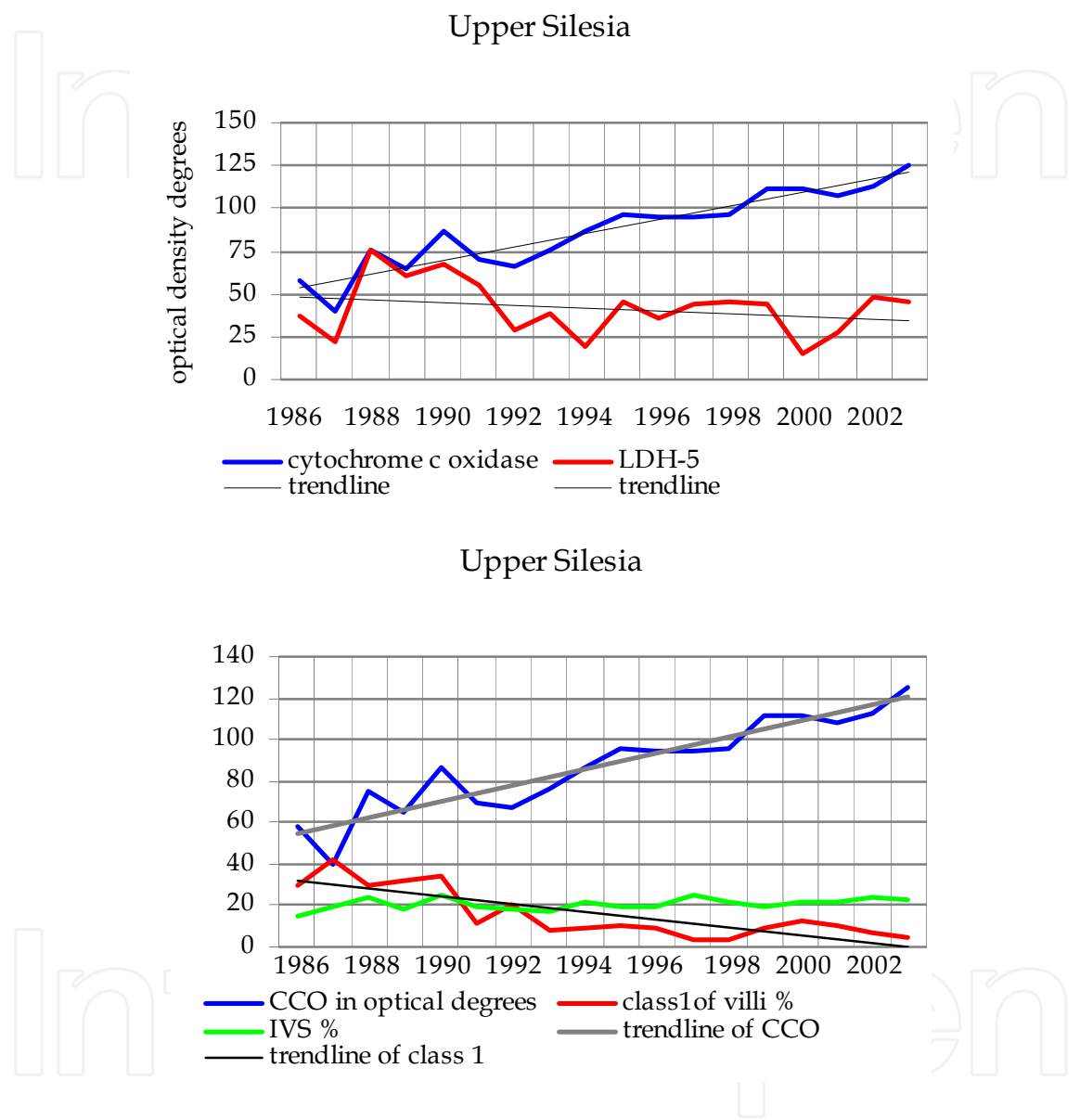


Fig. 9. The relationship of some placental characteristics for Upper Silesia region. The upper figure in clear manner shows inverse correlation between the activities of oxygen dependent cytochrome c oxidase in term of hypoxia-inducible LDH-5 isoenzyme. The lower one serves to clarify reverse dependencies of the cytochrome c oxidase activity and small dimensional terminal villi generation affiliated by decrease (in term of control material) intervillous space volume. Incredibly suggestive is elucidation of the cytochrome c oxidase activity increase during the research period on the background of improvement parameters of environmental conditions in Upper Silesia region (compare with fig. 10).

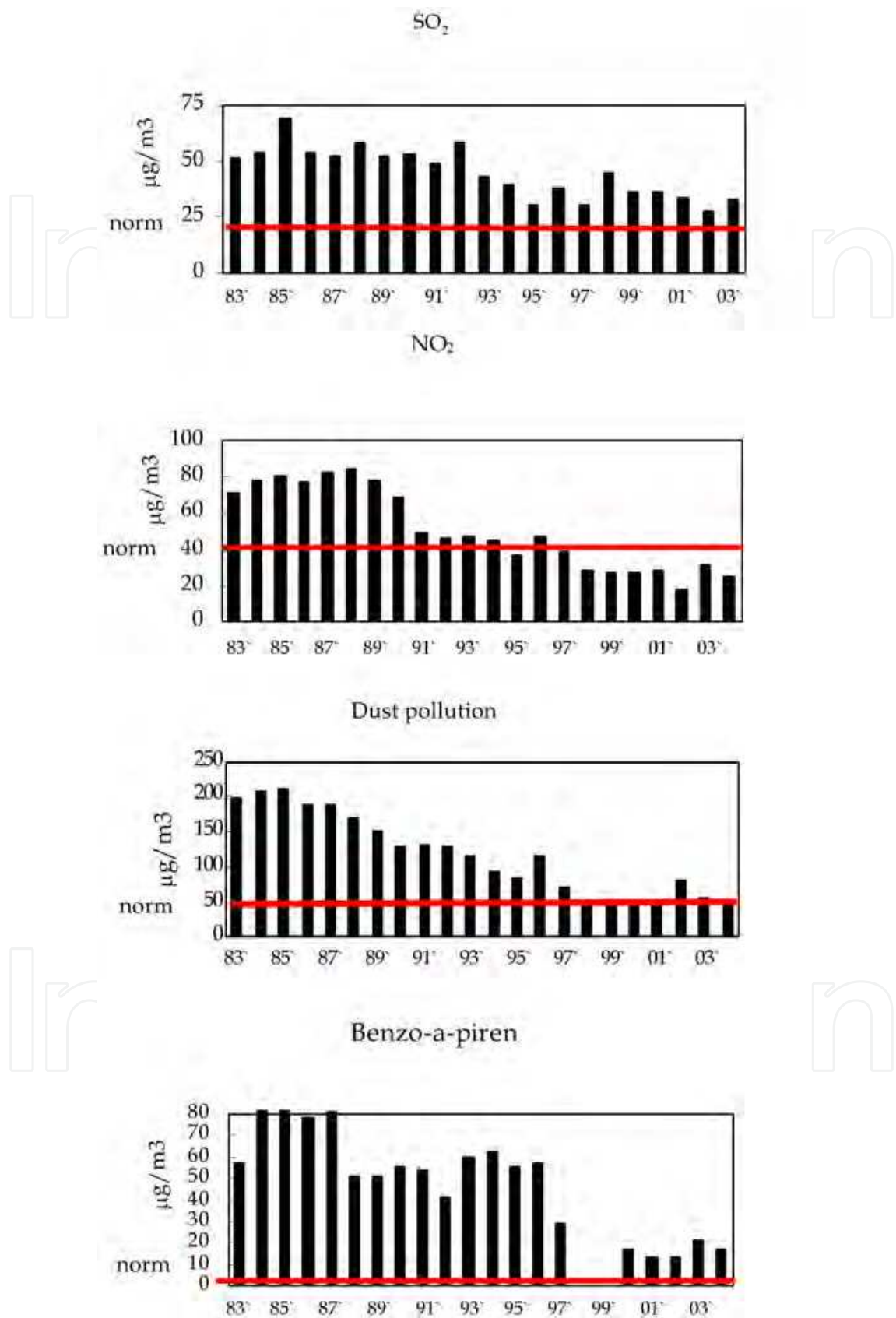


Fig. 10. The average annual emissions of SO_2 , NO_2 , dust and benzo-a-piren pollutants for Upper Silesia region in period 1983 - 2003.

	Copper Basin		Control		p value
	Male (n = 26)	Female (n = 24)	Male (n = 18)	Female (n = 20)	
Mothers age (years)	24.9 ± 1.0 (17-37)	23.2 ± 1.1 (19-35)	27.5 ± 1.1 (19-38)	26.7 ± 1.5 (17-38)	NS
Gestational age (weeks)	39.9 ± 0.2 (38-42)	40.2 ± 0.3 (38-42)	39.4 ± 0.2 (38-42)	39.6 ± 0.3 (38-42)	NS
Newborn`s birth weight (g)	3551 ± 90 <i>a</i> (2600-4150)	3360 ± 123 <i>b</i> (2400-3290)	3571 ± 122 (2900-4050)	3511 ± 92 <i>c</i> (2900-3970)	<i>a,b</i> p <0,05 <i>b,c</i> p <0,05
Newborn`s length (cm)	55.1 ± 0.5 (49-61)	54.3 ± 0.8 (48-60)	56.0 ± 0.6 (50-63)	54.9 ± 0.6 (48-59)	NS
Placental weight (g)	518 ± 16 <i>a</i> (350-720)	466 ± 29 <i>b</i> (330-690)	508 ± 22 (330-710)	446 ± 16 (350-570)	<i>a,b</i> p <0,05

Table 5. The clinical characteristics of the studied groups. Data are presented as mean ± SEM.; (range); NS, not significant; Kruskal-Wallis and post-hoc Dunn test.

The mother’s age and gestational age showed no differences between the studied groups. Birth weight was significantly lower for girls than boys but only from the Copper Basin region. Girls of the copper basin also had a significantly lower birth weight than girls from the control region. Similarly, neonatal length and placental weight were lower for girls than boys, but only the differences between the placental weight of girls and boys from the Copper Basin region were statistically significant.

According to many reports (Bleker et al., 1979; Kato, 2004), the average birth weight of boys is usually slightly higher than the birth weight of girls in a healthy population. Studies of the Carpathian newborns (low polluted control material) for over 18 years, showed no statistically significant differences between the birth weight of boys and girls (3490 ± 58.2; n = 108 vs 3443 ± 64.4; n = 119 respectively). However, in healthy newborns from high polluted areas, these differences were visible (Upper Silesia: boys 3369 ± 53.1, n = 102 vs girls 3189 ± 61.8, n = 95, p < 0.05; Copper Basin: see table 5). This may indicate a greater susceptibility of female fetuses to harmful environmental factors.

3.2 The concentrations of heavy metals and essential minerals in placentas and fetal membranes

The concentrations of copper, manganese and chrome in the placentas of the studied regions were found to be statistically different. An increase in the concentration of copper (34%) and a decrease of manganese (38%) and chromium (30%) in placentas from the Copper Basin, when compared to the control samples, was observed. For fetal membranes, the statistically significant increase of concentration of copper (84%), manganese (230%), chromium (58%) and mercury (60%) in samples from the Copper Basin, when compared to the control group, was found. The mean concentrations of trace elements in the placentas and fetal membranes of neonates of both sexes from studied regions are summarized in table 6.

Element	Placenta		Fetal membranes	
	Control (38)	Copper Basin (50)	Control (36)	Copper Basin (32)
Cu	2.15 ± 0.19 * (0.75-5.58)	2.89 ± 0.23 (1.02-7.84)	2.56 ± 0.17 *** (0.31-4.95)	4.71 ± 0.29 (0.98-10.71)
Zn	16.97 ± 1.31 (7.72-36.44)	16.24 ± 1.42 (7.81-38.01)	7.54 ± 0.34 (4.75-14.99)	8.81 ± 0.43 (3.67-18.50)
Mn	1.55 ± 0.34 * (0.18-7.86)	0.96 ± 0.21 (0.00-6.67)	0.27 ± 0.06 * (0.00-1.37)	0.89 ± 0.24 (0.16-3.82)
Cr	1.99 ± 0.28 * (0.59-5.55)	1.38 ± 0.36 (0.51-5.08)	0.92 ± 0.41 * (0.14-4.52)	1.45 ± 0.58 (0.41-7.09)
Pb	0.78 ± 0.10 (0.35-1.75)	0.80 ± 0.28 (0.17-3.85)	0.61 ± 0.19 (0.00-1.28)	0.64 ± 0.17 (0.11-1.90)
Hg	1.07 ± 0.25 (0.00-1.95)	1.11 ± 0.19 (0.41-1.99)	0.51 ± 0.29 * (0.00-2.13)	0.83 ± 0.19 (0.00-1.75)
Ca	1546 ± 420 * (416-6598)	3161 ± 928 (740-24100)	210 ± 10.2 (122-369)	313 ± 32.1 (106-922)

Table 6. The mean concentrations (in µg/g) of trace elements in the placentas and fetal membranes of neonates of both sexes from the Copper Basin and control regions. Data are presented as mean ± SEM.; (range). *p<0.05, ***p<0.001, Mann-Whitney U test.

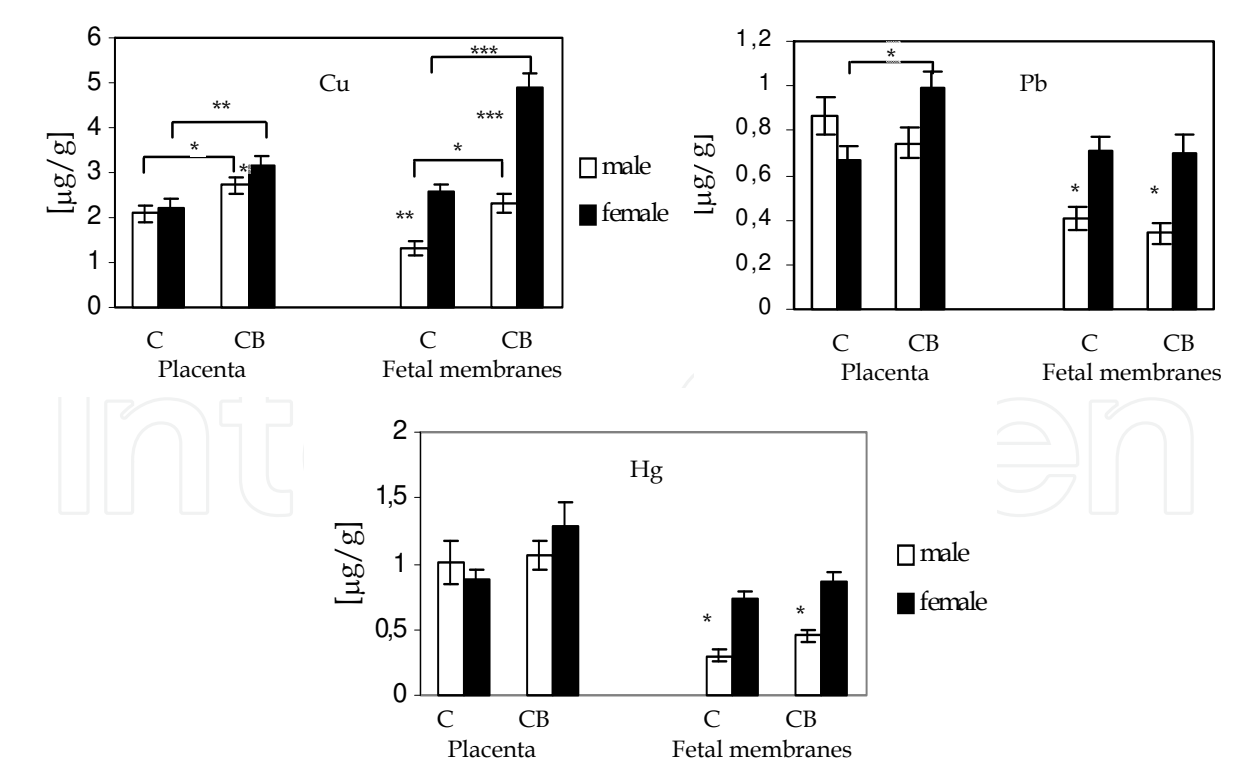


Fig. 11. Mean concentration ± SEM of copper, lead and mercury in male and female placentas and fetal membranes of Copper Basin (CB) and control (C) regions. *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis and post-hoc Dunn test.

The levels of elements such as zinc, manganese, chrome, mercury and calcium were higher in the placenta than in fetal membranes mainly in the control group. Conversely, the level of copper was twice as high in the fetal membranes than in the placenta, but only in the Copper Basin group. Lead levels did not differ between the placenta and fetal membranes.

A comparison between the afterbirths of male and female neonates from the Copper Basin and control regions showed interesting differences in the concentrations of copper, lead and mercury. Generally, the higher contents of these metals was found in the female versus male placentas from the Copper Basin. The concentration of copper was higher by 14% ($p < 0.05$), lead by 25% ($p < 0.05$) and mercury by 16%, as illustrated in figure 11. The controls' female placentas had significantly lower concentrations of the studied metals than female placentas from the Copper Basin. For fetal membranes, the statistically significant increase in the concentration of the studied metals in female samples when compared to the male ones, was found both in the Copper Basin and control materials, figure 11.

3.2.1 Copper

It is known that copper toxicity is an acute episode usually resulting from copper contamination of drinking water or other beverages. However, relatively little information is available about the effect of long-term exposure to copper on the human body. Copper is essential for the human body. As a basic bioelement, it is a component of numerous enzymes including cytochrome c oxidase (Yewey & Caughey, 1988). However, due to the high rate of copper bioaccumulation and the big extent of its anthropogenic activation, it causes a risk to the local environment via contamination. On the Copper Basin, this risk arises from the long-term operation and processing of copper ore. An excessive copper drop, when accompanied by other metals and contaminating compounds, leads to the degradation of all the ecosystems when it exceeds the level of its acceptable environmental concentration. In cells, copper accumulates mainly in the mitochondria and binds to DNA, RNA, and the nucleus. With its ability to bind with nucleic acids, copper can cause permanent changes in their structure and thus alter their biological properties (Kabata-Pendias & Pendias 1993). Copper and its ions have a very high affinity for sulfhydryl groups of various enzymes and a significant share in the creation of the reactive oxygen species that initiate the peroxidation of lipids in cell membranes, thus leading to the inactivation of enzymes associated with these membranes (Chan et al., 1982). Sokol et al. (Sokol et al., 1993), especially in the mitochondrial lipid peroxidation induced by copper toxic action, which is considered the reason of the cytochrome c oxidase activity decrease. The activity of this enzyme and glucose-6-phosphate dehydrogenase was significantly lower in the investigated placentas from the Copper Basin in relation to control materials, both in the placental villi and in the amniotic epithelium (Zadrožna, 2003). Moreover, the activity of these enzymes significantly negatively correlated with a high copper concentration in the placentas and fetal membranes from the Copper Basin. We observed negative correlations between: the cytochrome c oxidase activity of placental villi and placental copper concentration ($r = -0.2952$, $p < 0.05$), cytochrome c oxidase activity of amniotic epithelium and copper concentration in fetal membranes ($r = -0.3055$, $p < 0.05$) and glucose-6-phosphatase dehydrogenase activity of amniotic epithelium and copper concentration in the fetal membranes ($r = -0.3471$, $p < 0.05$). Additionally, the relationship between sex and the copper content in the placentas and fetal membranes was demonstrated. A significantly higher concentration of copper in female versus male afterbirths was observed.

It is reported that, during pregnancy, maternal serum has twice the level of copper found in it than normal healthy adults, which suggests an important role of this mineral during gestation (Krachler et al., 1999). The placenta can accumulate copper either from ceruloplasmin or from low molecular complexes (McArdle & van den Berg, 1992). The level of copper in the placenta is largely dependent on interactions with other minerals. Especially, zinc interacts directly with copper. The researchers propose that zinc may interfere with copper absorption by competing for binding sites on metallothionein, which regulate the transportation such a metals as zinc, copper and cadmium (Itoh, 1996; Milnerowicz, 1993). Animal studies report that zinc deficiency increases plasma copper levels (O'Dell, 1976, as cited in Massaro, 1997). Conversely, the high levels of zinc induce symptoms of a copper deficiency in laboratory animals (Klevy, et al., 1994; Oestreicher & Cousin, 1985). However, in our study, the levels of zinc in the placentas and fetal membranes did not differ between two studied regions. In addition to zinc, copper also interacts with calcium. The level in the placentas from the Copper Basin was two times higher than in the control. The studies of laboratory animals indicate that copper increases the loss of calcium from the bone (Wang & Bhattacharyya, 1993). Yet, on the other hand, high intakes of calcium and phosphorus lead to reportedly lower copper retention in the human body (Spencer et al., 1984, as cited in Massaro, 1997). Also, iron deficiency increases copper levels in the placenta (McArdle et al., 2008), but this metal has not been tested in our study.

3.2.2 Manganese and chromium

The next comparison of the Copper Basin and control materials allowed us to note the different concentration of manganese in placentas and fetal membranes. The placentas from the Copper Basin contained 38% less manganese than the control placentas while the fetal membranes from the Copper Basin were richer in manganese by 70% than the control ones. These similar proportions were observed relative to chrome in the studied materials. Overall, in the control material, the concentrations of manganese and chromium in placentas were relatively high, while they were low in the fetal membranes. In turn, in the material from the Copper Basin, the average concentrations of these metals in placentas and fetal membranes were similar. The above results from our observation are apparently an effect of the interactions between chromium, manganese and calcium. The limited research that exists indicates that calcium inhibits manganese absorption in laboratory animals (Van Barneveld & Van den Hamer, 1984).

Manganese is an essential element for growth and bone development. This confirms the positive correlation ($r = 0.6011$, $p = 0.02$) between the birth weight of girls and placental concentrations of manganese found in the material from the Copper Basin. The lower concentration of manganese and chromium in the placentas from the Copper Basin in relation to the control, and higher concentrations of these metals in the fetal membranes, may indicate on increase in the placental barrier permeability for chromium and manganese under conditions of prolonged exposure to environmental toxins.

3.2.3 Lead

It is universally accepted that lead represents a potentially toxic agent in humans. On the basis of numerous reports (Korpela et al. 1986; Lagerkvist et al. 1996), it is known that lead

can cross the placenta and have consequences upon the developing fetus. Many older papers have proved that high doses of lead can cause miscarriage (Ernhart, 1992), and epidemiological data indicated the negative effect of lead contamination during pregnancy on newborn children and during early childhood (Shukla et al. 1989). However, more recent reports (Rudkowski, 1999) pay attention to the fact that the already observed destructive results of lead in children were not considered as dangerous till now. Dietrich (Dietrich, 1987) and Needleman (Needleman, 1990) proved that the so-called low levels of lead found in the blood of pregnant women exposed to environmental lead could cause not only abnormal fetal development but also impaired postnatal mental development. It is very important to note that, during pregnancy, there can occur the mobilization of lead (together with calcium) from the bone, which has collected there as a result of prior accumulation, especially since the half-life of lead in bones takes approximately 20-30 years (O'Flaherty, 1995; Gulson, 1997). Currently, on the basis of numerous epidemiological studies concerning the concentration of lead in children's blood, that may cause harmful effects, is calculated at 100 µg/l (Dutkiewicz, 1993). According to data by the Foundation for Children from the Copper Basin (Strugała-Stawik, 1999), the percentage of children tested in this area, in which the concentration of lead in blood was higher than 100 µg/l, was respectively 16.0%, 13.6% and 11.6% in the years 1995-1997. This data also showed a higher concentration of lead in the blood of boys than girls. It is well documented (Iyengar & Rapp, 2001; Lagerkvist et al., 1996; Loiacono et al., 1992), based on studies of the lead levels in maternal blood, umbilical cord blood and placenta that, that lead does not accumulate in the placenta and that the placenta does not impede, to any significant extent, the transfer of lead from the mother to the fetus. It is therefore concluded that the placenta is not suitable material for use in the monitoring of environmental lead exposure. This is in line with our research findings. In our study, the concentrations of lead in the placentas and fetal membranes from the Copper Basin did not demonstrate differences in comparison to the control, although the previously cited data showed that lead mikointoxication was a serious problem in this territory. No differences in lead content between placenta and fetal membrane samples in both groups were also observed. A lack of expected differences in the lead concentration in the investigated materials may also be the result of lead interactions with other metals. In the human body lead is accompanied usually by calcium. Lead and calcium interact in a negative manner. Calcium deficiency raises lead toxicity, and an adequate calcium intake decreases lead toxicity. In human infants, lead absorption decreases as dietary calcium increases (Ziegler et al., 1978 as cited in Massaro, 1997). Also, higher calcium intakes decrease lead absorption in human adults (Blake & Mann, 1983).

However, analyzing separately male and female afterbirths from the Copper Basin, a higher lead concentration ($p < 0.05$) in placentas as well as in fetal membranes of female neonates was visible than in the male ones. This would suggest the higher ability of the lead accumulation by a female afterbirth in dangerous environment circumstances.

3.2.4 Mercury

Mercury is a ubiquitous environmental toxicant. There are three main forms of mercury: elemental, inorganic, and organic compounds (methyl and ethyl mercury). Humans are exposed to all of its forms and even relatively low doses of mercury containing compounds can have serious adverse neurodevelopmental impacts (Choi et al., 1978). Mercury exposure has a potentially negative effect on fetal development (Ask et al., 2002), because it can

interfere with the developing nervous system. Mercury exposure for the fetus and nursing infants comes both from mercury stored in the woman's body prior to pregnancy, and from mercury to which the woman is exposed during pregnancy and breast feeding. The organic mercury can easily pass through the placental barrier and accumulates in the fetus. Some recent researches (Rudge et al., 2009; Stern & Smith, 2003) indicate that the cord's blood mercury concentration was almost twice as higher than the maternal. The results obtained through examining maternal blood, placental tissue and umbilical cord blood from an urban region showed a higher total mercury content in the placenta than in maternal and cord blood (Tsuchiya et al., 1984). The opposite results were obtained earlier by Hubermont et al. (Hubermont et al., 1978), who showed the highest levels of mercury was in blood samples from newborns when compared to the maternal blood and placenta from rural areas of Belgium. Based on these results, Iyengar and Rapp (Iyengar & Rapp, 2001) conclude that higher mercury content in the umbilical cord blood and placental tissue than in maternal blood indicated the presence of the transplacental pathway of mercury. The placentas from urban areas have slightly more ability to retain mercury in their tissues.

The results of our research showed no differences in the placental total mercury concentrations, but not in fetal membranes, between a polluted and rural region. The concentration of mercury in the fetal membranes from the Copper Basin were higher ($p < 0.05$) than in the control. Also, only female fetal membranes had a significantly higher mercury content than the male membranes, both in the material of the Copper Basin and in the control group. Methylmercury toxicity studies conducted on laboratory animals have shown variation among sexes. Female mice revealed a higher resistance to methylmercury acute toxicity than the males (Yasutake et al., 1990), and upon life-long exposure to methylmercury, male mice and rats manifested neurotoxic symptoms earlier than the females (Mitsumori et al., 1990).

4. Mineral deposits and calcium levels in high polluted placentas

The placentas from Upper Silesia and the Copper Basin of both sexes were richer in mineral deposits than the control, figure 12. In female placentas from the Copper Basin, the big amounts of mineral deposits were found more often than in male placentas, although the differences were statistically non-significant.

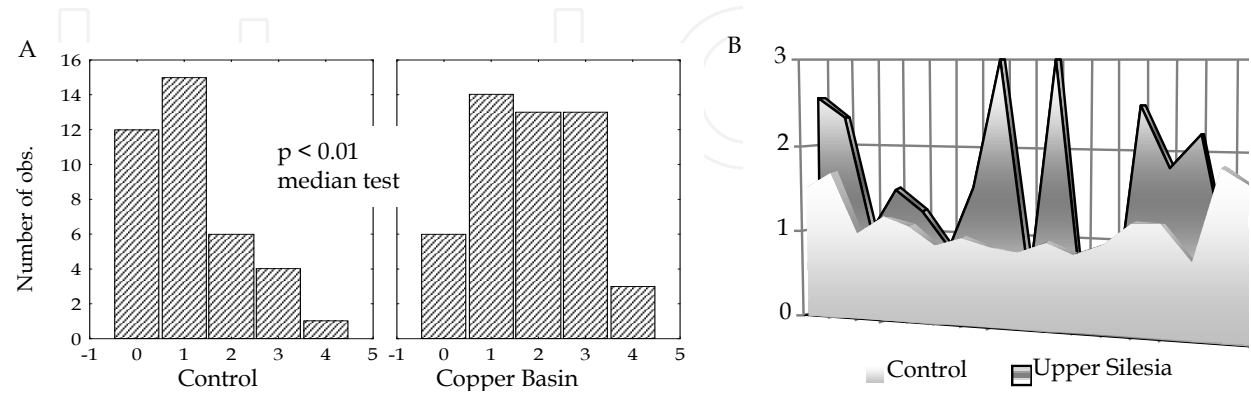


Fig. 12. A. The frequency distribution of mineral deposits occurrence in the Copper Basin and control placentas. B. The mean abundance of mineral deposits in placentas from Upper Silesia and control regions during the years 1986-2003; $p < 0.01$, median test. Scale: from 0 to 4 in ascending gradation order.

Mineral deposits usually occur in anomalous fibrous and hyaline connective tissue structures in stem and terminal villi as well as in blood vessel walls and cytotrophoblastic islets. Mineral deposits appear yellow or brown and stippled, and occur in irregular quantities. In the tissue sections they are visible in the form of transparent crystals, figures 13, 14.

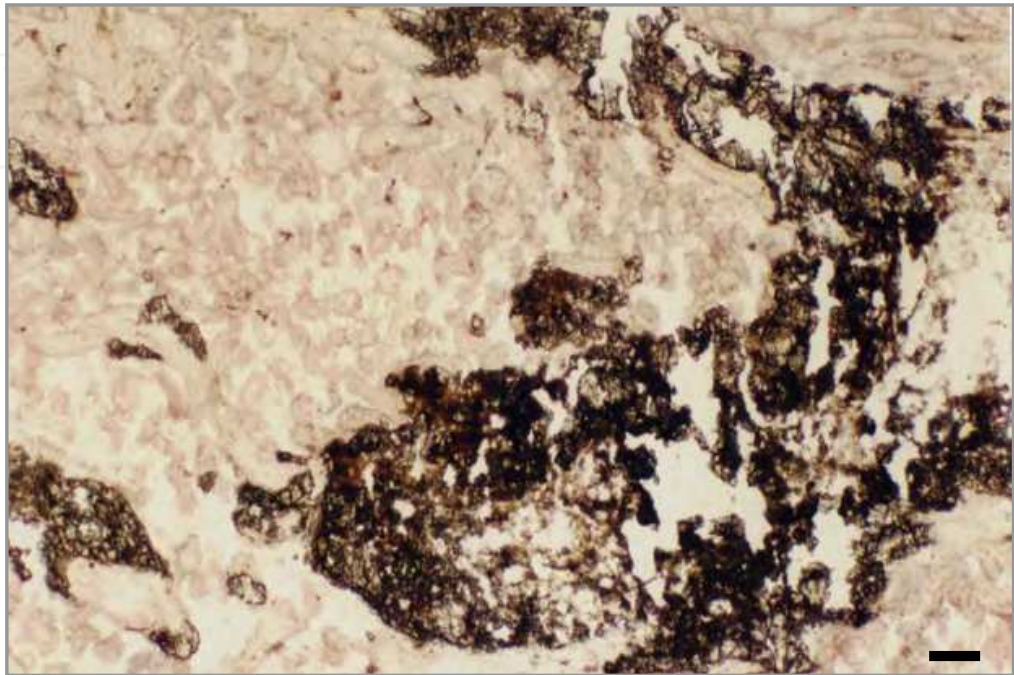


Fig. 13. Cryostat cross-section through a full term placenta from Upper Silesia. The giant mineral deposit; and in the background histochemical reactions for cytochrome c oxidase. Bar: 50 μ m.

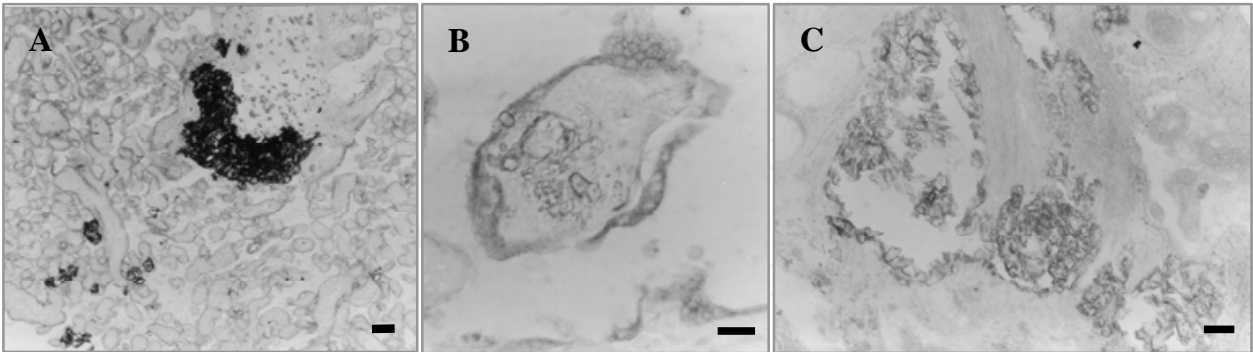


Fig. 14. Cryostat cross-section through a full-term placentas collected in the Copper Basin territory. A. Numerous of mineral deposits deposited within the cytotrophoblastic island and the intervillous space. Bar: 100 μ m. B. Mineral deposits within the placental villous. Bar: 20 μ m. C. Mineral deposits in the chorionic plate. Bar: 50 μ m.

Necrosis of placental tissues and excess of calcium ions are believed to be one of the reasons for mineral deposits originating. The researches of the placentas collected from the aluminum smelter territory (Zamorska, 1982/1983)) have revealed adverse effects of fluorine compounds on the condition of placentas and newborns, and also a significant

increase in the content of mineral deposits and richness of fluoride in these deposits. The placenta, as it is known, limits the passage of fluoride ions from the maternal blood to fetal blood, binding them mainly with calcium or magnesium ions and forming insoluble salts (Shen & Taves, 1974). In this way the placenta protects the fetus from an excess of fluoride when the maternal fluoride intake is high. These observations confirm the results of studies on the fluoride and calcium distribution in the placenta (Chlubek et al., 1998). The high positive correlation between fluoride and calcium concentrations in the marginal part of placentas of women residing in an area with a relatively low water and air fluoride content was found.

In our studied materials, the chemical analysis proved that there was twice as much concentration of calcium in the placentas from the Copper Basin than in the control ($p < 0.05$). Moreover, we found a strong positive correlation ($r = 0.4877$; $p < 0.000$) between the calcium concentration and the mineral deposits, and a negative correlation between calcium and a newborn's length ($r = -0.543$; $p < 0.05$).

It is known that the calcium excess can cause the alteration of cell metabolism as a consequence of interaction with a series of cellular trace elements (Miller & Groziak, 1997), and in extremal cases it causes cell death by necrosis or apoptosis (Leist & Nicotera, 1998). In cytotoxicity induced by the calcium ions there is a substantial participation of mitochondria (Kang, 1997). They are able to cumulate the calcium ions and in this way reduce their concentration in cytoplasm, and consequently prevent their toxic activity (Rutter et al., 1993). On the other hand, the calcium ion excess in mitochondria leads to their dysfunction and thereby to the decrease of ATP production (Bernardi, 1996). The statistically significant negative correlation ($r = -0.3224$; $p = 0.011$) between cytochrome c oxidase activity of placental villi and placental calcium concentration in the Copper Basin samples was found. Moreover, the strong negative correlation between activity of cytochrome c oxidase of the placental villi and mineral deposits was observed both in the Copper Basin ($r = -0.2416$; $p < 0.001$) and Upper Silesia materials ($r = -0.2468$; $p = 0.012$).

In examining the placentas from low polluted regions, we observed that cigarette smoking by pregnant women is also associated with the occurrence of large amounts of mineral deposits in the placenta.

5. Conclusion

The hypothesis that the morphology of the feto-placental unit is sensitive to the impact of all environmental pollutants is fully confirmed here, and the presented changes are of a dose-dependent effect type. We found that enzyme histochemistry is a very useful tool for studying placental physiology and for metabolic and morphological connections. Our presented placental studies are the only ones, as far as we are aware, which covers the longest monitoring period and because of it we seriously rely on the strength of our arguments.

Our prolonged studies of the effect of environmental chemical pollution on the biochemical and microanatomical organization of the human placenta showed that this organ, when influenced by toxic factors, undergoes changes. The primary change consists in histochemically detectable deterioration of the oxidative enzymes activity in the villous syncytiotrophoblast as compared with the activity shown by placentas from sites of low

pollution. Especially the measurement of cytochrome c oxidase activity is a good indicator of oxygen availability in utero-placental environment and thus a good marker of energy supply for the fetus. This consequently results in placental hypoxia and in compensatory increase in numerical density of the small dimensional villi.

The presented morphological measurements do not meet the requirements of today's stereology (Mayhew, TM., 2008, 2009). It is worth noting, however, that in the 1980s, the reciprocal presuppositions of contemporary placental stereology were shaped. The data collected for so many years using the same assumptions provides a strong basis for the outcome of comparisons between environments.

Our study also showed an interesting variation among sexes. In the highly polluted environment, female afterbirths accumulated more copper, lead and mercury. Simultaneously the female newborns had a significantly lower average birth weight than male infants, but there was no direct correlation with a higher content of studied metals in the placenta. One interesting observation is a positive correlation between the mean weight of newborns and manganese content, which in the placentas from the Copper Basin was significantly less.

The human placenta is not a sensitive indicator of environmental contamination of toxic trace elements because, as it is clear from many studies, it is not a barrier for metals such as lead or mercury. If, however, despite this, the concentration of toxic trace elements in placentas increases, this information could be used to diminish postnatal exposure of the fetus or maternal exposure during subsequent pregnancies, as suggested Iyengar (Iyengar & Rapp, 2001).

The systematic collection of reliable information on concentrations of lead, cadmium, copper, themselves recognized as major contributors to environmental pollution, in the human body is the most effective method for evaluating the effectiveness of activities aimed at protecting the environment and the health of residents.

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