ORIGINAL ARTICLE

Placenta – organ important for fetus and interesting for the rise of the Attention Deficit Hyperactivity Disorder Syndrome – interdisciplinary study

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Abstract

OBJECTIVE: This work is aiming at broadening knowledge about placenta and giving evidence that lead penetrates through transplacental barrier. We have intended to find further possible reasons of the rise of Attention Deficit Hyperactivity Disorder (ADHD) and to suggest ways of preventing development of this syndrome.

METHODS: For revealing presence of lead in placenta and umbilical cord blood we used histochemical methods, scanning and transmission electron microscopy, Energy dispersive spectroscopy (EDS) analysis of element composition and infrared spectroscopy.

RESULTS: We are presenting new findings that emphasize importance of Hofbauer cells. These cells have high phagocyting activity and form filter regulating entering lead into umbilical cord blood and thus influence possibility of the ADHD rise. We found positivity on lipids in placenta. Importance of this finding consists in the fact that lead is lipophilic metal and tissue containing lipid is available path for the transport of lead. We explained why just striatum is affected with toxic action of lead in case of ADHD syndrome. We have also shown that more blood elements circulate in umbilical cord blood than in a common circulation.

CONCLUSIONS: High number of patients with the ADHD syndrome inspired us to suggest establishment of centers where these children would be registered. Staff of specialists consisting of psychologist, physician and physicist (responsible for application of methods of early diagnosis) will take care of the development of their health conditions and further treatment.

INTRODUCTION

Several years ago placenta was considered a biological waste. Nowadays we are aware of the fact that placenta is a source of valuable information (Baglan *et al.* 1997; Lafond *et al.* 2004; Foltinová *et al.*; Reichrtová *et al.* 1998a,b). Picture of placenta after the childbirth is a "mirror" of history of pregnancy. This organ reveals still unknown things also about the origin of ADHD. After the childbirth a newly born child bears for the postnatal life beside other metabolites important for life also heavy metals including lead. Presence of this neurotoxic heavy metal depends on the environment where mother had lived during all her life. Influence of polluted environment may leave changes on microscopic structure as we have pointed out in our recent works (Foltinová 2000, 2006, 2009).

It has not passed a long time since petrol enriched with lead has not been used anymore and consequences of this enrichment are already here (Bailey *et al.* 2002). One of the consequences is influence of lead on the rise of ADHD in children. In organism lead can cause irreversible changes in a tissue and directly endanger developing fetus. Hemo-encephalic barrier is well permeable for lead – hence nothing prevents lead from direct attacking brain structures of the developing fetus (Goyer 1990; Needleman & Bellinger 1991; Drtílková 2007; Baranowski & Norska-Borówka 1996; Grandjean 1978; Šimera 2008).

In this work we give complex analysis of sequence of changes in placenta and their effect on umbilical cord blood and fetus. This blood forms "a bridge" for transport of lead towards the fetus. From the physical point of view it is a difference whether mother's blood flows or squirts into placenta. Also this fact is explained in this work. We succeeded in obtaining these results owing to new methodology and progressive technology. From the point of view of ADHD development we consider necessary depistage of children with this diagnosis for the healthy development of the further generations. One must keep in mind that relative amount of absorbed lead in a child is much higher than in an adult. Moreover, the faster growth of cells and their faster dividing in a child organism means increased risk for damaging genetic material.

MATERIAL AND METHODS

We prepared and evaluated sections from excisions of placentas of 119 healthy patients. Concurrently we examined umbilical cord blood of 50 patients by means of infrared spectroscopy using KBr pellet making technique. Moreover, from 3 000 child patients hospitalized at Clinic of Child Psychiatry of Child Faculty of Hospital with Polyclinic in Bratislava during years 2003–2006 there were 120 patients with ADHD. We investigated this set of patients. In our investigation we abode rules of medical ethics.

Excisions from placenta were fixed in AFO – alcohol-formol-acetic acid in the ratio 12:6:1. On the 7μm thick paraffin sections we carried out the following histological staining methods:

- Hematoxylin-eosin
- Lendrum "acid-picro" Mallory
- Una Tanzer "acid-picro, indigocarmin-orcein"
- New methodical approach after Foltinová, which is combination of Mallory and Parker method for proof of lead with the software program Imago Pro Plus 45 Media Cybernetics Inc. assisting to microscope Olympus BX-50 with Sony three CCD. Positivity on lead is manifested by turquoise green colour.

Excisions from placenta were for proof of lipids fixed in Baker calcinated formol and stained by Sudan black method and Nile blue method.

Umbilical cord blood was investigated on smears stained by method after May Grünwald-Giems Romanovsky. Excisions from placenta were for scanning and transmission electron microscopes prepared by double fixation with glutaraldehyde (200 mmol/L) and osmium tetroxide (OsO_4 40 mmol/L) that were buffered by phosphates with pH 7.25. Excisions were dehydrated by alcohols and dried at the critical point of CO_2. The method for evaluation of excisions in scanning electron microscope was described in our previous paper (Foltinová *et al.* 2007). Ultrathin sections of excisions from placenta were prepared methodically for transmission electron microscopy.

The following devices were used for the evaluation:

- Light microscope Reichart Polyvar (Germany) at magnifications 180–1 500×
- Scanning electron microscope PHILIPS CM 20 (Holland) at magnification 365×
- For EDS analysis of element composition SEM JEOL JXA 840A at magnifications 400–7 000×
- Infrared spectrometer SPECORD M10, Carl Zeiss, Jena (Germany)
- Transmission electron microscope JEM-EX 1200, JEOL, Tokyo (Japan) operating with 80 kV accelerating voltage with magnification 30 000×.

RESULTS AND DISCUSSION

In our recent papers we discussed finding of lead in syncytiotrophoblast from various aspects. We pointed out that calcium and lead show positivity in the same places of the surface part of syncytiotrophoblast (Foltinová *et al.* 2007). In our later work (Foltinová *et al.* 2010) we emphasized changes of structure of syncytiotrophoblast when placenta is mature. Now we are presenting new findings that demonstrate importance of Hofbauer cells in the placental villi which have high phagocyting activity. These cells contain in their cytoplasm various amount of phagocyted particles including lead. These particles have a shape of small grains that are present in cytoplasm. Turquoise green ring around densely gath-

Fig. 1. Placental villus, stained by method for proof on lead after Foltinová. Erythrocyte with positivity on releasing lead in the lumen of fetal vessel: on the tip of large arrow. Erythrocyte of mother's blood with positivity on lead: on the tip of small arrow. Positivity on lead is stained turquoise green. Magnified: 650×.

Fig. 2. Placental villus, stained by method for proof on lead after Foltinová. Gathering of Hofbauer cells with positivity on lead: tip of thick arrow directed upwards. Single Hofbauer cells with positivity on lead: tips of thin arrows. Mother's erythrocyte with positivity on lead: tip of thick arrow directed downwards. Positivity on lead is stained turquoise green. Magnified: 650×.

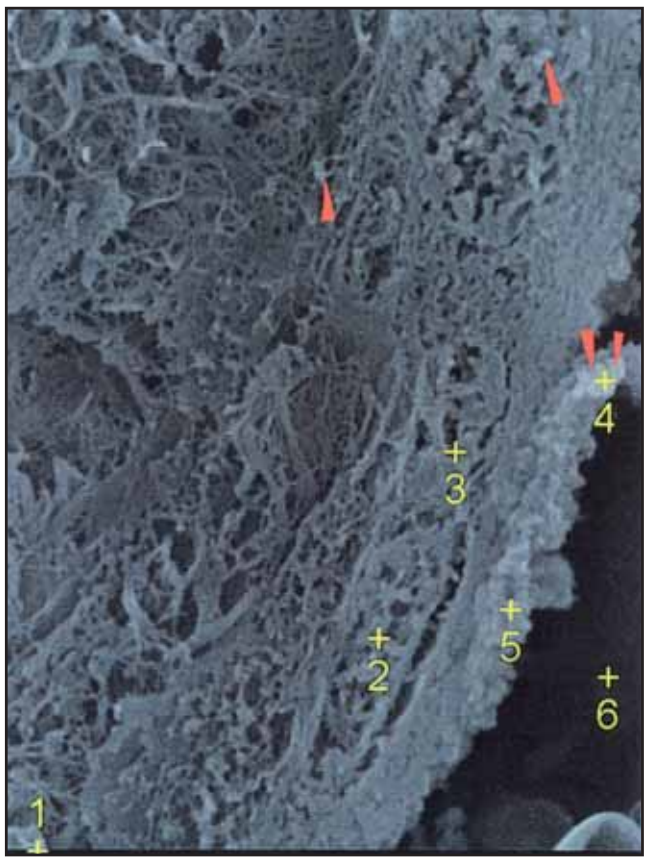

Fig. 3 (left). Placental villus diagnosed in scanning electron microscope, evaluated by EDS method in the depicted six points the choice and evaluation of which is discussed in the text. Gathering of Hofbauer cells: point "+4": tips of two thin arrows placed near to "+4" directed downwards. Single Hofbauer cells: on the tips of thin arrows directed upwards. Magnified: 1 500×.

ered grains of lead in form of its compounds means positive finding of lead that deserves attention. Positions of these cells are interesting (Figure 2). They take place around vessels in the placental villi. Figuratively speaking: Hofbauer cells due to their positions and function "act as detectives" in wait for lead. They guard that it may get into umbilical cord blood in the least amount. These cells form filter regulating entering lead into umbilical cord blood. Amount of Hofbauer cells and their functional aspect depend on immunologic state of mother as well as of environment in which pregnant woman lived. Psychical stress is an enemy of these cells, what is important fact for prenatal life of individual. Fragile fetus in uterus is sensitive to consequences on the rise of which this heavy metal with toxic effect participates.

New method for proof of lead after Foltinová (2006) helps to visualize trace amounts of lead in a tissue. We have found that negativity on lead in excisions of pla-

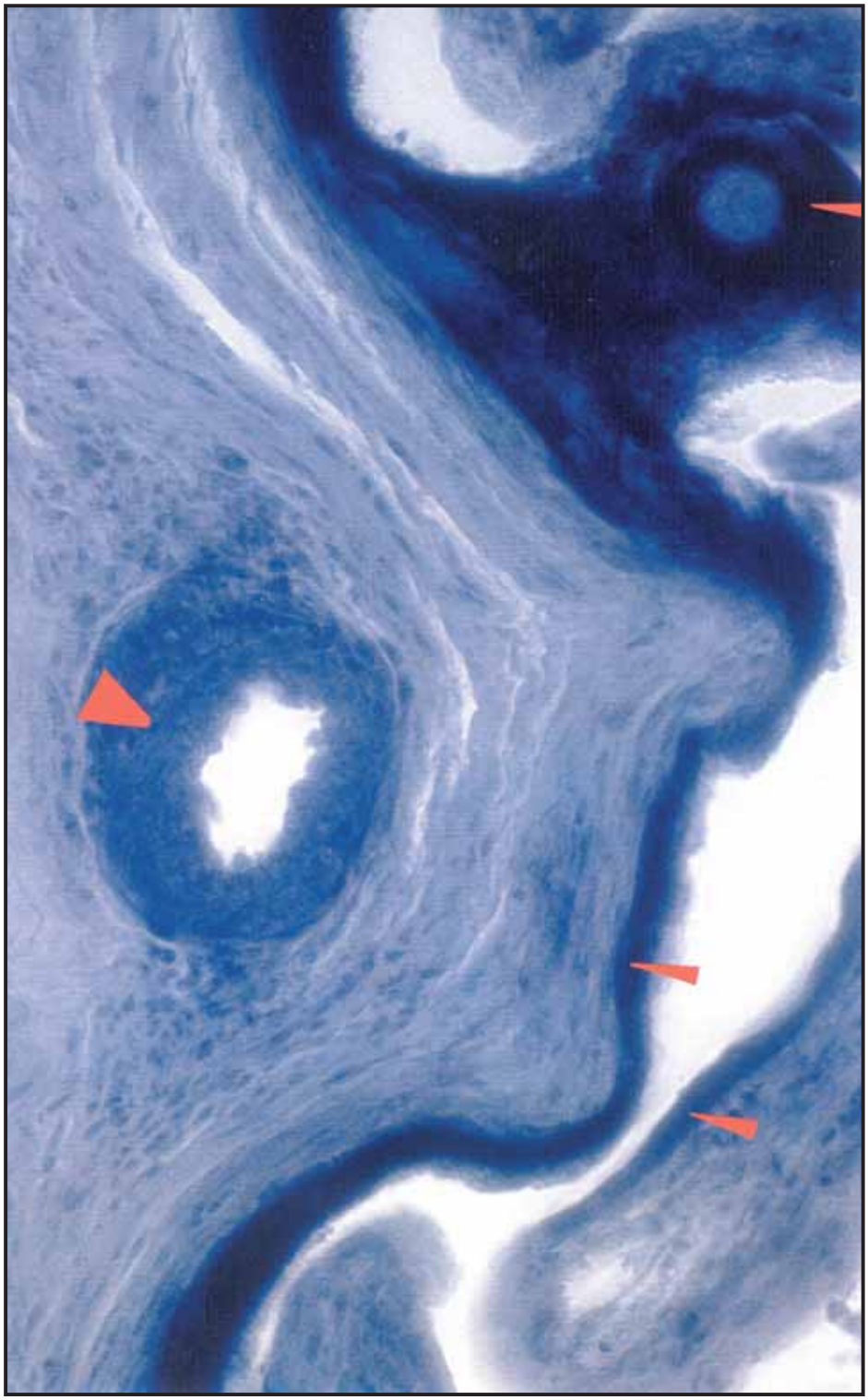

Fig. 4. Placenta, positivity on lipids. Stained by Nile blue method. Significant positivity in syncytiotrophoblast: thin arrows directed from right to left. Wall of the placental vessel: thick arrow. Significant positivity on the basal pole of syncytiotrophoblast. Magnified: 220×.

centa need not necessarily mean negativity on lead in fetus. Concurrently with histological methods we had therefore to pick up suitable physical techniques in order to avoid this possible gap of certainty. We decided for the SEM/EDS method and infrared spectroscopy. SEM/EDS: "Scanning Electron Microscopy with X-ray Microanalysis" is a technique used to identify the elemental composition of a sample or small area of interest on the sample. During EDS a sample is exposed to an electron beam inside a scanning electron microscope. These electrons collide with the electrons of the sample causing some of them to be struck out of their orbits. The vacated positions are filled by higher energy electrons that emit x-rays. By analyzing the emitted x-rays the elemental composition of the sample can be determined. X-ray spectra may be accumulated within minutes (Krištín *et al.* 2005). Beside EDS method we utilized in our investigation also method based on

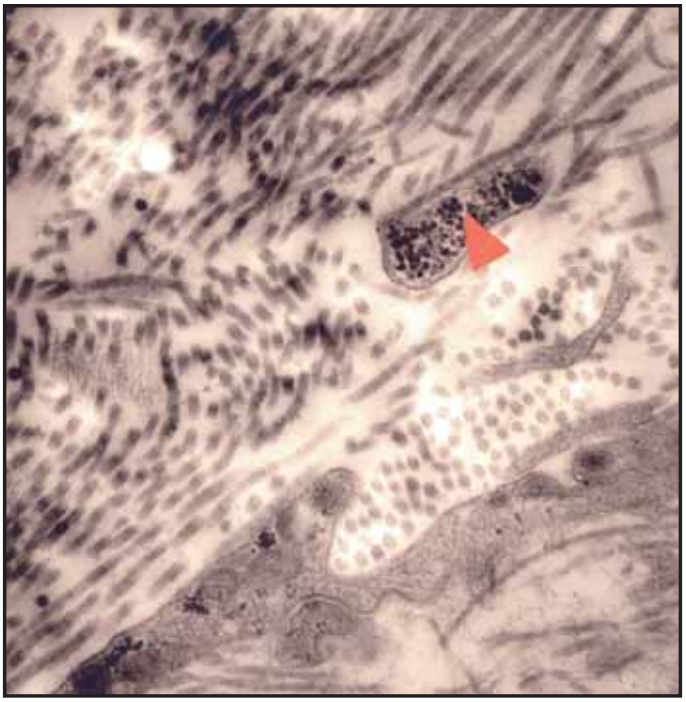

Fig. 5. Placental villus: a part of transplacental barrier in transmission electron microscope. Cross section of umbilical cord vessel, lumen of which is filled with variously dense blood elements: thick leaning arrow directed from right to left. Vessel of placenta: thick arrow. Magnified: 30 000×.

infrared spectroscopy. We used this method as a tool for obtaining detailed information about chemical compounds in which pollutants of interest occur in the sample (Bentley *et al.* 1968, Hollas *et al.* 2005). In this way we got information enabling us to discuss probable origin of pollution.

From the physical point of view there are two important factors influencing lead transport in placenta. The first factor is connected with the amount of lead carried by blood and the second factor is connected with the possibility of lead being trapped in placenta. These factors depend on the way of blood flow in placenta. In places with slow blood circulation a less amount of lead appears but this lead can be more easily captured. In places where blood squirts – what happens at the entrance of mother's blood into placenta – higher amount of lead appears and this lead is worse captured. Situation is complicated also due to hidden places of lead in case of wrong choice of investigated excisions. Moreover, Hofbauer cells act as lead "traps" localized in the villi of placenta and therefore not all lead from mother's blood is transported through transplacental barrier into the lumen of vessels in which blood of the fetus circulates. Just the lead of blood circulating there affects possibility of the ADHD rise. Beside Hofbauer cells (Figure 2) also mother's blood erythrocytes occurring in intervillous space of placenta showed positivity on lead (Figure 1).

For the proof of cumulation and transport of lead we used energy dispersion analysis (EDS) of element composition in excisions of placenta (Figure 3). Point analysis of elements was carried out in six chosen places of the tissue. These places were chosen so as to check high occurrence of lead in placenta determined by histochemical method. To avoid erroneous evaluation one place was picked up in an empty space forming thus a background (place denoted as "+6"). The sites with suspected highest levels of lead occurrence are denoted as "+4": gathering of Hofbauer cells, "+5": syncytiotrophoblast. For comparison three "neutral" sites were picked up for evaluation and denoted by signs "+1", "+2", "+3". EDS analysis confirmed highest values of lead in gathered Hofbauer cells and in syncytiotrophoblast and decreasing content of lead with increasing distance from syncytiotrophoblast in sites "+1", "+2", "+3". Occurrence of lead in "+6" point was negligible what demonstrates validity of evaluation.

We examined presence of further interesting compound in mature placenta. There we found positivity on lipids (Figure 4). This is important finding because lead is lipophilic heavy metal. Tissue containing lipid is available path for the lead transport. Positivity was in the outlines of villi. This finding is a warning for obstetrician who might donate a newly born child with lead in case of prolonged childbirth. This warning ensues from the fact that long lasting childbirth may lead to hypoxic attack what affects function of Hofbauer cells. In such attack changes of permeability of cellular membrane appear, hence Hofbauer cell lets lead pass into umbilical cord blood. Small amount of lead is sufficient for serious consequences in the organism of a newly born child who has not mature microscopic structures including brain and therefore is more vulnerable towards the rise of ADHD. In children suffering from ADHD diminishing of brain volume of 3–8% was found in comparison with healthy children. Total reduction concerned both gray and white mass. Microscopic structure of striatum is sensitive to perinatal hypoxic attacks. In striatum dopamine is important for influencing psychomotorics, attention and type of behavior (Drtílková 2007). Lead transported through hemo-encephalic barrier damages dopamine paths of the middle brain, e.g. striatum. Mainly basal ganglia are very sensitive against hypoxia. Attention must be paid to the fact that myelin sheaths are maturing only in the postnatal stage. In their biochemical structure lipoproteins are involved. This is a convenient basis for toxic action of lead and its compounds. This is explanation why just striatum is affected with toxic action of lead in case of ADHD.

In transmission electron microscope we have shown a part of the transplacental barrier inevitable for lead transport from syncytiotrophoblast to a vessel in the placental villus. We can see stripes of various electron microscopic density and umbilical cord vessel in the lumen of which blood elements of various stages of maturity occur (Figure 5). Conspicuous gathering of

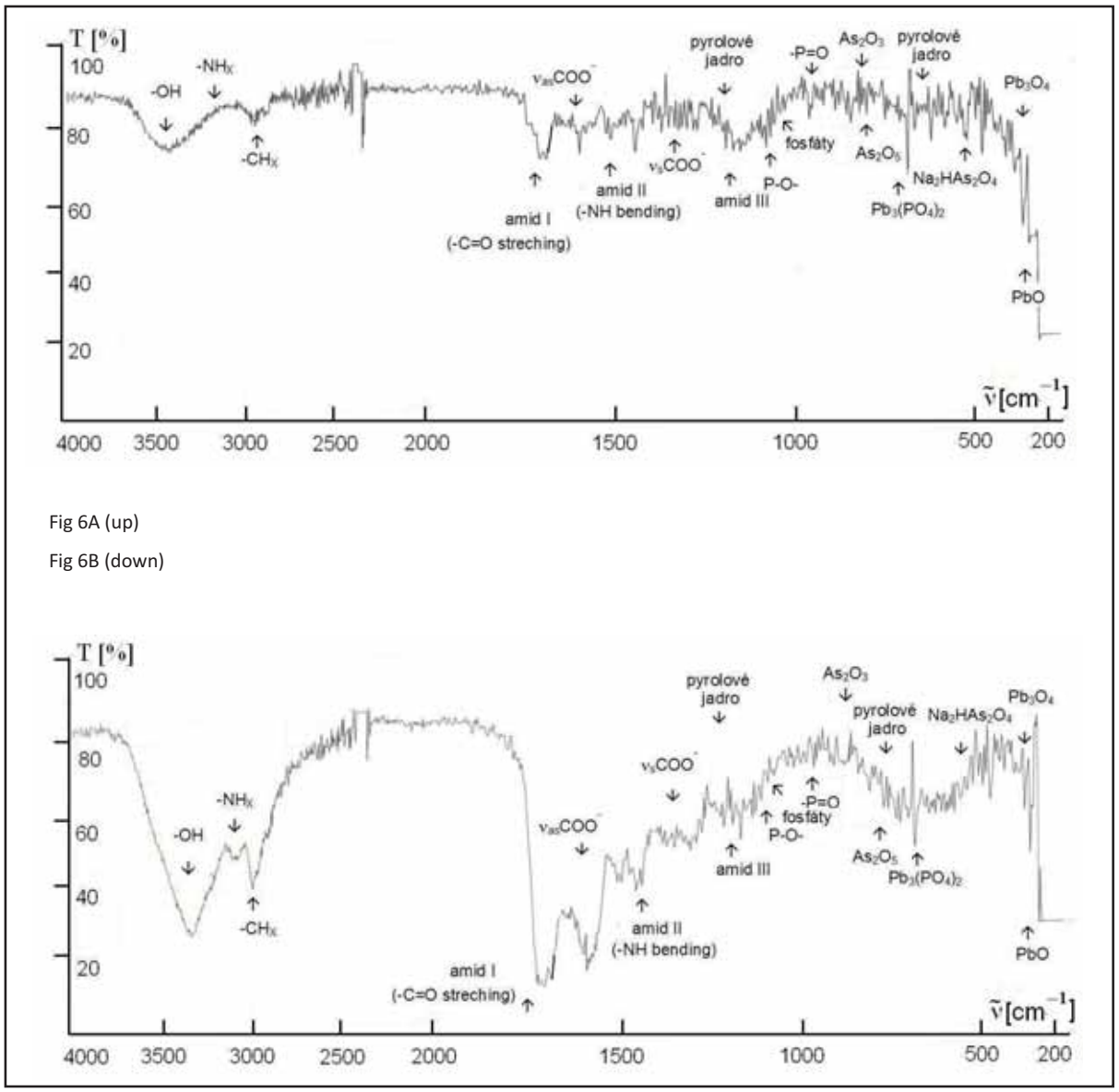

Fig 6A (up)

Fig 6B (down)

Fig.6. Placenta – positivity on lead. Diagnosed by infrared spectroscopy for various times of dwelling in environment polluted with lead when petrol enriched with lead was used. Expression "pyrolové jadro" is pyrolle ring; expression "fosfáty" is phosphates. A (upper graph) – placenta of 26 year old mother, B (lower graph) – placenta of 41 year old mother.

blood elements in this vessel gives evidence about the fact that there circulate more blood elements in umbilical cord blood than in a common circulation. This demonstrates that not only in the blood smear, as we indicated in our recent paper (Foltinová *et al.* 2010), but already before the childbirth waning of erythrocytes occurs. This is a demand of physiology of the developing fetus. Closely before the childbirth there is a surplus of decaying erythrocytes in umbilical cord vessels of placenta. This phenomenon in a newly born child may be one of the reasons of the rise of newly born child icterus. This icterus is of various intensity in clinical picture. In the newly born child physiologic changes of blood picture closely before and after the childbirth appear. They consist in a change in distribution of erythrocytes when erythrocytes decay and blood picture accommodates to changed conditions because after the childbirth lungs are already functional and the inner medium of the subtle individual does not need such number of erythrocytes as in the intrauterinary development. In umbilical cord blood we succeeded to prove by means of infrared spectroscopy presence of lead in form of $Pb_3(PO_4)_2$, PbO, Pb_3O_4. Moreover, we recorded arsenic in form of oxides (As_2O_3 and As_2O_5) and Na_2HAsO_4. From these compounds namely $Pb_3(PO_4)_2$ is most important for us since we expect it to come from bone deposits where lead substitutes calcium in hydroxyl apatite (Pounds 1991). Oxides of arsenic and lead may be consequences of processing in glass industry or they may be secondary forms produced in organism. Owing to their age all women of our set were exposed also to exhaust gas of vehicles using petrol enriched with lead.

Hence they respired $Pb_3(NO_3)_2$ that is soluble in water and dissociates to $Pb^{2+}(aq) + 2NO_3 - (aq)$. We assume that the nitric acid might be utilized in various physiologic processes. Bivalent cathion of lead may react with further reactants and thus change places of its occurrence. In literature bones and teeth are considered as places of final deposition of lead compounds in the organism (Pounds 1991, Foltinová 2006). We therefore assume that $Pb_3(PO_4)_2$ comes from these deposits and is a final product of biotransformation of lead in human organism. Since $Pb_3(PO_4)_2$ is rather weakly soluble in water we assume existence of enzymatic mechanism facilitating dissociation of phosphate group from this compound. Due to decay of $Pb_3(PO_4)_2$ lead is again free for forming further types of compounds. This is important finding and in relevant scientific databases we did not find reference to occurrence of $Pb_3(PO_4)_2$ in umbilical cord blood. A danger exists that in prenatal stage, when decay of erythrocytes occurs, lead may be released and menace the surrounding. In case of umbilical cord blood these compounds are mainly oxides. Particular form depends on the degree of blood oxidation.

On the spectra we can see various measures of occurrence of decay products, mainly pyrols and phosphates. We can say with certainty that children of women whose age was in time of childbirth lower than 26 years, i.e. lived for shorter time in the polluted environment, had lower concentration of these metal compounds in umbilical cord blood than children of women who lived for 41 years in this polluted environment (Figure 6).

CONCLUSIONS

Phagocyting Hofbauer cell plays important role in reducing amount of lead penetrating into umbilical cord blood in form of various compounds. Fetus draws from this blood beside nutrients and oxygen also unwanted elements with toxic effect. Lead belongs to this type of elements. Fragile developing microscopic structures of particular organs of fetus are affected with this blood. Already prenatally they obtain hallmark for postnatal life. By means of broad spectrum of laboratory diagnostic methods we succeeded in identifying Hofbauer cells, single and in groups, their position in relation to transplacental barrier and occurrence of lead. One of diagnostic methods is polarization microscopy, but this method is not specific and alone is not sufficient for proof on lead. We have shown that only in case when histochemical results are positive on lead and positive on lead are also results of infrared spectroscopy in umbilical cord blood, we can speak about positivity on lead relevant for discussion about the rise of the ADHD.

High number of patients with the ADHD syndrome leads us to suggest establishment of centers where these children would be recorded and their parents encouraged to let them to be examined and cured. Staff of these centers should consist of psychologist, physician and physicist responsible for application of methods for determining early diagnosis. They should appeal on parents to get rid of shyness since not the mother but polluted environment is responsible for the illness of a child. Our results are motivation for taking both umbilical cord blood and mother's blood immediately after the childbirth and examine them for the presence of lead in order to begin early prevention of ADHD.

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