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Heavy metals nanoparticles in fetal kidney and liver tissues

Antonietta M. Gatti[1], Paolo Bosco[2], Francesco Rivasi[1], Sebastiano Bianca[3], Giuseppe Ettore[3], Luigi Gaetti[4], Stefano Montanari[5], Giovanni Bartoloni[6], Diego Gazzolo[7,8]

[1]Laboratory of Biomaterials, Dept. of Specialistic Surgeries, University of Modena and Reggio Emilia, Modena Italy, [2]Institute for Research on Mental Retardation and Brain Aging IRCCS Oasi di Troina, Troina, Italy, [3]Department of Maternal, Fetal and Neonatal Health G. Garibaldi Hospital, Catania, Italy, [4]Institute of Anatomy, Mantova Hospital, Mantova, Italy, [5]Nanodiagnostics Research Laboratory, Modena, Italy, [6]Institute of Perinatal Malformative Disorders, G. Garibaldi Hospital, Catania, Italy, [7]Department of Pediatrics and Neuroscience Giannina Gaslini Children's University Hospital, Genoa, Italy, [8]Neonatal Intensive Care Unit, C. Arrigo Children's Hospital, Alessandria, Italy

TABLE OF CONTENTS

1. ABSTRACT

The proliferation of the nanotechnologies with the production of engineered nanoparticles presents a dilemma to regulators regarding hazard identification mostly for human health. We investigated the presence of inorganic micro and nanosized contamination in fetal liver and kidney tissues by Field Emission Gun-Environmental Scanning Electron Microscope (FEGESEM) innovative observations. An observational study in 16 fetuses, complicated (n=8) or not (n=8) by neural tube defects, whose mothers obtained the authorization for abortion between 21-23 weeks of gestation was carried out. Heavy metals concentrations in maternal blood were undetectable. FEGESEM assessment showed particles of iron, silicon, aluminum and magnesium in different tissues analyzed. The mean size and the number of the foreign bodies detected in kidney and liver tissues were higher in NTD fetuses as well as the number of total particles ($P<0.05$, for all). The present study shows first the presence of xenobiotic, nanoscaled contamination, not detectable in maternal blood in fetuses. Data are suggestive and open-up a new clue for further investigations to elucidate the relationship between pollution at nanoscale stage and multiorgan damage.

2. INTRODUCTION

Improvement in technological processes, requiring higher and higher temperatures, generates a source of pollution also at nanoscale level that can be inhaled or ingested with nanocontaminated food and can reach any organ being alveoli or colon barriers efficient enough (1,2). The rapid proliferation of the nanotechnologies with the production of many different nanomaterials, including engineered nanoparticles, ambient ultrafine particles and biological nanoparticles, presents a dilemma to regulators regarding hazard identification (3). Nanoparticles are commonly defined as materials that have structural features with at least one dimension of 100 nanometers or less. Such materials typically possess nanostructure dependent properties (e.g., chemical, mechanical, electrical, optical, magnetic, biological), which make them potentially risky to human health including the ability of entering the body – *via* air and liquid suspension - and exhibiting biological and toxicological activity associated with their nanostructure (3).

The potential risk for human health concerns their ability to evade the natural defenses of the organism and to interact directly with proteins, enzymes, DNA etc. since

Table 1. Chemistry, size and number of particles detected in kidney and liver tissues in fetuses complicated by neural tube defects (NTD) and spontaneous miscarriage (SM)

Particles characteristics	**NTD (n=8)**	**No-NTD (n=8)**
	Size (μm)	**Size (μm)**
Fe	3.9 +/- 3.3	3.7 +/- 1.5
Fe-Cr-Ni	6.5 +/- 3.7	4.8 +/- 2.5
Si	6.7 +/- 5.8	0
Al	3.0	0
Mg	7.5 +/- 6.0	0
	Particles (n°)	**Particles (n°)**
Fe	19	3
Fe-Cr-Ni	7	7
Si	15	0
Al	1	0
Mg	7	0
Total*	49	10

Note: Data are expressed as means ± SD. (*P<0.05)

they fall within the same size range. Of note, there is growing evidence that environmental contamination, including heavy metals, might occupy a key role in higher risk for Central Nervous System (CNS) malformations such as neural tube defects (NTD) and spina bifida (4-7). Humans and experimental data reported that heavy metals such as arsenic, lead, cadmium, and mercury are neurotoxic and may have a teratogenic role. Pregnant women (10=5) drinking lead contaminated water were more likely to have NTDs in their offspring (8-14). In this setting, data on possible nanoparticles presence in fetal tissues such as liver and kidney as a result of overt or not maternal blood contamination are to date lacking.

Therefore, the aim of the present study was to investigate the presence of heavy metal nanoparticles contamination in kidney and liver fetal tissues in absence of any detectable contamination in maternal blood.

3. MATERIALS AND METHODS

The study was performed at our tertiary referral Centers for Obstetrics and Perinatal Malformation Disorders between December 2004 and July 2006. We carried out an observational study in 16 pregnancies complicated (n=8) or not (n=8) by NTDs such as spina bifida whose mothers (mean age 22 years) obtained the authorization for abortion between 21-23 weeks of gestation.

Exclusion criteria were: multiple pregnancies, chromosomal abnormalities, and maternal exposure to alcohol, cocaine and smoke.

The study protocol was approved by the local ethics committee and pregnant women gave informed and signed consent.

3.1. Sample preparations

Autoptic samples of liver and kidney collected from 16 fetuses were immediately fixed in 10% formalin, dehydrated in ascending concentration alcohols and embedded in paraffin. Table 1 shows the list of the analyzed cases. 5-10 micron thick sections were cut by means of a Leica microtome (Leica RM 2125RT, Nussloch, Germany). The thinner sections were prepared for histology, the thicker ones were deposited on an acetate sheet, de-paraffined with xylol and mounted on an Aluminum stub. To remove the paraffin, the samples were covered with a few drops of xylol and 98%-alcohol and, a few seconds after, the excess of liquid was slid along the sheet edge and absorbed in blotting paper.

Then they were inserted inside the chamber of a Field Emission Gun-Environmental Scanning Electron Microscope (FEG-ESEM Quanta, by FEI Company, Eindhoven, The Netherlands) equipped with an Energy Dispersive System (EDS by EDAX, Mahwah, NJ, USA).

The FEG-ESEM was selected for its high sensitivity and for the peculiarity to work also in environmental conditions (and not in vacuum), so that no preparation of the sample (dehydration, carbon or gold coating to make the sample electron-conductive) is necessary, thus avoiding further contamination and the consequent formation of artifacts. The EDS microanalysis system collects the X-rays generated by sample's surface hit by the electron beam, sorts and plots them by energy, and allows to identify the elements that produced the peaks in this energy distribution. The system can identify the elemental composition of the materials imaged for all elements with an atomic number greater than boron. Most elements can be detected at concentrations of order 0.1%.

Tissues' morphological observations and chemical analyses require to place the biological section on a substrate of acetate because, under the Energy Dispersive Spectroscopy, it emits only minor signals for carbon and oxygen, ever-present elements in any biological specimen. When the section is very thin, the contribute from the acetate substrate is just added to that from the biological specimen and does not affect the measure carried out on the inorganic debris, eventually present in the biological samples. This kind of samples can be observed in different modalities: in low vacuum; from 20 to 30kV, in secondary and in backscattered electron mode, with spots from 3 to 5. The backscattered modality allows showing materials with higher atomic density than the biological matrix. Since these xenobiotics have a micrometric and nanometric size it is necessary to divide the section in frames at low magnification (300x) and observe every frame at increasing magnification up to 30000-60000x to detect nanosized

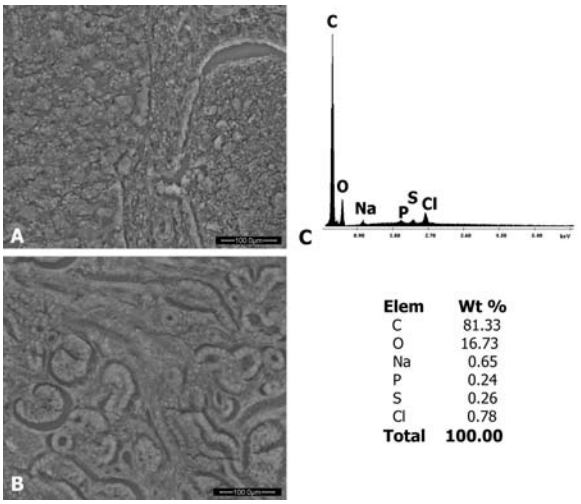

Figure 1. Microphotograph of liver (A) and kidney (B) fetal autoptic samples. C reports the EDS spectrum of a healthy fixed biological tissue with the semiquantitative elemental analysis of the normal tissue.

particles. The minimum size of the particles inside the biological substrate, detectable by the FEG-ESEM in low vacuum mode and in a well-preserved tissue, is 10nm.

When inorganic bodies are observed, they are measured, photographed and then the x-ray microprobe is focused on it to obtain the EDS spectrum. For every sample a list of the particles found with their size, morphology and chemical composition is carried out.

3.2. Metal particles measurements

Maternal blood for metal particles measurements was collected from cubital vein at admission to the unit and immediately centrifuged at 1500 g for 10 min and the supernatants stored at -70°C. Measurement was performed by using a spectophotometer (Varian SpectrAA 200 Atomic Absorption Spectrophotometer, Palo Alto, USA). The following component were assessed: sodium, calcium, potassium, lead, chromium, silicon, aluminum, copper, nickel, iron, zinc, barium, magnesium, phosphorus, chlorine, titanium, carbon, oxygen, sulphur, bismuth.

3.3. Statistical analysis

Clinical data were expressed as mean ± SD. Maternal blood heavy metals concentrations, and the number nanoparticles detected in study-groups comparisons were performed by Student t-test and Mann-Whitney U two-sided test when the data were not normally distributed. Comparison between proportions was performed with Fisher's exact test. A value of $P<0.05$ was considered significant.

4. RESULTS

Heavy metals concentrations in maternal blood were undetectable in all samples collected at admission to hospital (data not shown).

Histological investigation at 4 to 100 magnifications showed the absence of any pattern suggestive of altered cells and tissue morphology.

4.1. FEG -ESEM microscopy patterns

Observations at magnification 100-1000x showed no presence of inorganic entities in the biological tissue. As expected a normal chemical composition of the tissues after fixation has been found by means of an x-ray microprobe of an Energy Dispersive System (EDS by EDAX). In particular, kidney and liver tissues showed the presence of several elements such as carbon, oxygen, sulphur, chlorine and calcium that were homogeneously distributed in agreement with previous observations. (15,16).

Observations at magnification 1000-60000x showed particles in different tissues that were analyzed by EDS to obtain their elemental composition on the basis of the highest elemental peak.

No significant differences ($P>0.05$, for all) in the distribution of chromium, zinc, nickel, copper, titanium, lead, barium, bismuth and tin, in NTD and control fetuses have been found (Table 1). Conversely, iron, silicon, aluminum and magnesium distribution was ($P<0.05$, for all) higher in NTD fetuses. The mean size and the number of the particles detected in kidney and liver tissues are reported in Table 1. There were no significant ($P>0.05$, for all) differences between groups regarding particle size whilst the number of total particles found in NTD group was significantly higher than in controls. No significant ($P>0.05$, for both) difference in the number of aluminum and of stainless and steel particles, defined as alloy of iron, chromium and nickel have been found. The number of iron, silicon and magnesium particles was significantly higher ($P<0.05$, for all) in NTD than in controls (Table 2).

Normal morphology of healthy liver and kidney tissues is reported in Figure 1 (Figure 1 -panel A and B). The EDS spectrum shows that healthy tissues contain ions of carbon, oxygen, sulphur, phosphorus and calcium (Figure 1- panel A, A1, B,B1). Instead in NTD tissues, micro and nanoscaled xenobiotics are found. They appear whiter than the background tissue since they are composed of atoms heavier than the biological substrate.

4.2. Special features

Fig.2 reports some identified findings (lighter particles) in the biological substrate (Figure 2). Fig.3 shows a cluster of nanoparticles, probably nanocrystals (for their needle shape) of Bismuth in a cavity surrounded by the tissue (Figure 3).

Figure 4 shows three different examples of Iron-based particulate matter (Figure 4). They present different compositions: the first are nanoparticles composed of Iron, Silicon and Aluminum (A1, A2), the second one (B1, B2) are nanoparticles composed of Iron, Chromium, Nickel, Copper, Manganese, namely stainless steel, and the third one (C1, C2) are nanoparticles composed of Iron, Phosphorus and Calcium. The different composition witnesses a different origin of the pollutants.

Table 2. List of the chemical composition of the particles detected in kidney and liver tissues in fetuses complicated by neural tube defects (NTD), and in no-NTD complicated (no-NTD) and statistical significance (P)

Particles (n° cases/total)	NTD(n=8)	No-NTD (n=8)	P
Fe	8/8	3/8	0.02*
Si	7/8	1/8	0.01*
Cr	6/8	4/8	0.60
Zn	6/8	2/8	0.13
Al	6/8	0/8	0.006*
Mg	5/8	0/8	0.02*
Ni	4/8	2/8	0.60
Cu	4/8	1/8	0.28
Ti	2/8	1/8	1.0
Pb	3/8	0/8	0.20
Ba	3/8	1/8	0.56
Bi	1/8	0/8	1.0
Sn	1/8	1/8	1.0

*Values with P<0.05

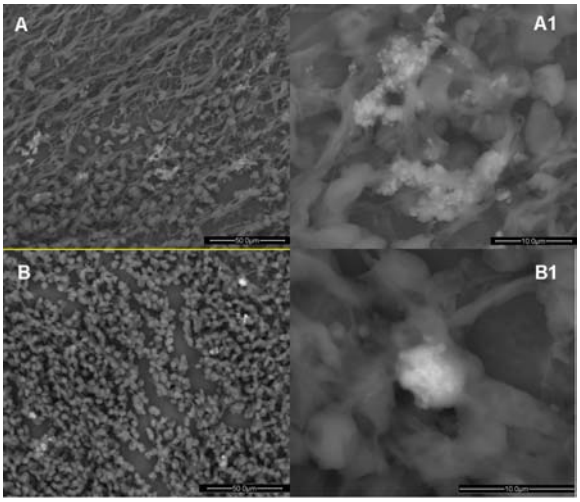

Figure 2. Morphological images of some liver (A, A1) and kidney (B, B1) fetal tissues at different magnifications (50micron and 10micron marker). The whiter debris represent the inorganic particulate matter identified in the internal organs.

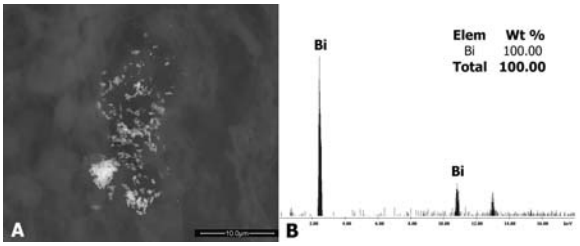

Figure 3. Particular of a liver sample where a cluster of Bismuth nanoparticles was identified.

5. DISCUSSION

The present study provides evidence that in fetuses complicated or not by NTD, such as spina bifida, nanoparticles are present both in kidney and liver tissues in absence of any detectable concentration in maternal blood. Furthermore, iron, silicon, aluminum and magnesium distribution and number of particles were higher in NTD fetuses.

The presence of heavy metal nanoparticles in fetal district warrants consideration: i) all NTD fetal tissues investigated were contaminated; ii) particles patterns are expression of environmental pollution; iii) all fetuses were dead at abortion procedure in absence of any respiratory activity. All together, the present findings suggest that fetuses' pollution derives from maternal blood contamination, reasonably related with mother assumption of contaminated food and water, leading to heavy metal nanoparticles transport from maternal to fetal bloodstream and finally to kidney and liver tissues. An additional *via* of contamination may be represented by respiratory tract: it has been recently reported that pollution at nanoscale stage (100 nanometers or less) can be inhaled through alveoli barrier due to a passive mechanism and finally into systemic circulation (1,2). On the basis of the present findings it can be argued that fetal liver and kidney tissues, through placental unit, could reasonably constitute a privileged bulk of nanoparticles storage in polluted cases. Further investigations on whole fetal organs are needed to clarify whether nanoparticles storage occurred by selective uptake mechanisms. The sources of this contamination may reasonably be related to maternal life-style and environment pollution as recently reported for artificial milks (17).

FEG-ESEM microscopy pattern showed an objective evidence of xenobiotics contamination in fetal kidney and liver tissues with a typical inflammatory response. This refers to the presence of calcium-phosphate precipitates and iron (15). The mechanism responsible of the formation of calcium-phosphate precipitates is controversial since it is not known whether this reaction is coupled with iron precipitation. The formation of calcium-phosphate precipitates implies that calcium and phosphorus ions are subtracted by the biological substrate (extra and intracellular) thus determining a reduced availability of these ions for the formation of important molecules like ATP and ADP. Although calcium-phosphate precipitates were of endogenous origin, it is uncertain for iron precipitates whether iron could come from the corrosion of the iron or stainless steel particles. This phenomenon can occur during the chemical degradation of stainless steel, in which iron ions in a non–equilibrium state are released and can immediately combine with ions of the extracellular matrix thus altering local pH and inducing metabolic alterations with metallo-protein formation (18). These products are not biodegradable/bioresorbable and can

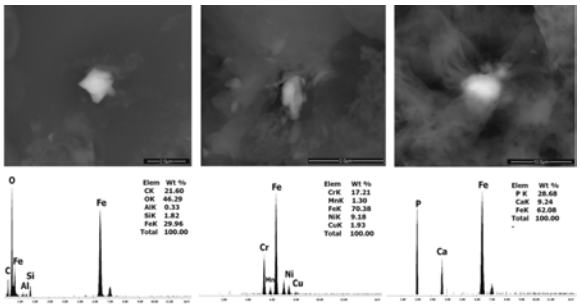

Figure 4. Microphotographs of three different types of Iron-based xenobiotics. The EDS spectra (after digital substraction of the normal tissue's spectrum) clarify that that the first debris contains Carbon, Oxygen, Iron, Silicon and Aluminum. The second one contains Iron, Chromium, Nickel, Copper and Manganese (a type of stainless steel) and the third one Iron, Phosphorus and Calcium. Three different chemical compositions mean 3 different sources.

create additional destabilization of the local cellular metabolic behavior. So that, bearing in mind that xenobiotics contamination can activate a cascade of events mimicking cytokines activation superimposable to perinatal infection (19,20). It is tempting to correlate the presence of nanoparticles pollution with the inflammatory reaction that can lead to the occurrence of preterm delivery that, to date constitutes one of the main issues of debate. In this setting, further investigations are needed.

In the present investigation we also found, in fetuses, the presence of other heavy metals whose effects on fetal CNS development can be of relevance. It has been previously reported that materials containing lead or copper or chromium are CNS chemically toxic (10,13,21). Crystals of bismuth, chromium, aluminum, copper, are recognized as not biocompatible/biodegradable and chemically toxic (3). Therefore the possibility that NTD fetuses, since from earliest post-conceptional stage, were exposed to environmental pollution, chemically toxic on CNS development is consistent.

Finally, we also found that in maternal blood of NTD fetuses heavy metal concentrations were undetectable at a stage when particles were already present in fetal kidney and liver tissues. The main explanations might be due to: i) technological and physical limits of the assessment methods used (i.e. limit of sensitivity of the assay); ii) the fact that heavy metal assessment in maternal blood might be dependent on pollution exposure modalities (i.e. length, frequency and timing of the exposure); iii) different maternal/fetal cardiac output that allow a reasonable heavy metal dilution in maternal bloodstream whilst fetal tissues such as kidney and liver may act as privileged site of storage.

In conclusion, the present study offers additional support on correlation between intrauterine and environmental pollution at a nanoscale level. The findings open-up to further investigations aimed at elucidated the mechanisms trough which nanoscaled particulate matter contamination could trigger CNS disease.

6. ACKNOWLEDGEMENTS

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Abbreviations: NTD, neural tube defect; FEG-ESEM, Field Emission Gun- Environmental Scanning Electron Microscope; EDS, Energy Dispersive Spectroscopy; CNS, Central Nervous System

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Send correspondence to: Antonietta M. Gatti, Laboratorio dei Biomateriali, Dipartimento di Chirurgie Specialistiche, Testa e Collo, Università di Modena e Reggio Emilia, via Campi 213/A- 41100 Modena, Italy, Tel: 39-0-592055701, Fax: 39-0-592055687, E-mail: antonietta.gatti@unimore.it