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Increased frequency of micronuclei in mononucleated lymphocytes and cytome analysis in healthy newborns as an early warning biomarkers of possible future health risks

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ABSTRACT

Impact of intrauterine development on health risks during adolescence and adulthood still needs to be investigated. The aim of study was to compare genome damage in newborns and mothers using the cytokinesis blocked micronucleus assay, nuclear division index (NDI), and centromere fluorescence in situ hybridization. The study was performed on 92 mothers and their newborns. Results showed that micronucleus frequency in binuclear T-lymphocytes (MNBN) in newborns was significantly lower than in mothers but higher in mononuclear T-lymphocytes (MNMONO). The NDI in the mothers was significantly higher than in the newborns. In newborns with <2500 g birth weight, NDI was similar to the mothers'. Mothers have significantly more centromere negative micronuclei than newborns. A significantly higher NDI and MNBN was found in newborns with ≥2 MNMONO/1000 than in newborns with <2 MNMONO/1000. It is suggested that MOMN and NDI might be good candidates for biomarkers of health risks in newborns.

1. Introduction

Current biomedical investigations of the causality between transplacental exposure and lifelong health risks are entering into a new era in which multidisciplinarity is opening up new frontiers in the application of methods and interpretation of data. Newborns with transient metabolisms and excretion systems between the fetal period and adult age require a specific approach in the evaluation of environmental health risks and the side effects of diagnostics and therapy. Knowledge on genome damage in newborns and the biomarkers used to predict increased health risks during childhood or adulthood is still very limited. Available data reveals that genome damage in newborns does not necessarily imply genome damage in their mothers or the presence of higher susceptibility during intrauterine development [1,2]. The continuous increase in childhood cancer incidences, neurodevelopmental disorders (autism and attention deficit hyperactivity disorder), testicular cancer in young men and breast cancer in younger age groups [3,4,5] are said to have their origin in intrauterine gene–environment interactions of non-genome mechanisms.

Pregnant women are exposed to a number of agents in their environment which are known or suspected to be genotoxicants. Newborn exposure outcomes have been shown to be the result of the interaction of possible intrauterine exposures and the combined action of the mother's, as well as of the fetal and placental metabolic systems. Xenobiotics to which the fetus can be exposed from the mother's intake of food, water or air, as well as maternal infections and drug treatment during pregnancy, may have a significant impact on the disturbance of developmental biological pathways [6,7].

The micronucleus (MN) assay is the only biomarker which detects both clastogenic and aneugenic effects of xenobiotics on the genome [8]. This method is standardized and validated as

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predictive for increased cancer risk [9]. It is a sensitive method used in the biomonitoring of newborns, children and adults [2,10,11]. Even though MN frequency was for decades primarily measured on binucleated T-lymphocytes using the in vitro MN assay [12] it was shown that, the scoring of MNMONO, cells escaping mitotic block in the cell culture, could give valuable information on genome damage [13–15].

The micronucleus frequency of in vitro divided MNBN reflects accumulated genome damage correlated to individual exposure. However, the MNMONO reflects genome damage in in vivo divided lymphocytes and hematopoietic stem cells [8,13,16]. Apart from that, the ratio between binucleated and mononucleated cells, the NDI gives insight into the immunological competence of an organism, as the lymphocyte mitosis initiation depends on several intrinsic factors such as, for example, interleukin levels [17]. Furthermore, the micronucleus assay enables the detection of other markers of genome damage, such as nuclear buds (NB) and nuclear plasmatic bridges (NPB) which originate from dicentric chromosomes, and the process of eliminating amplified DNA [18].

Thus, increased MN frequency in newborns in MNMONO, MNBN, NDI, NB and NPB are important biomarkers that can measure complex intrauterine stress with possibly long term health consequences.

This study is based on the Rhea mother–child cohort in Crete, Greece (http://www.enrieco.dk/Enrieco.Cohort.Show.asp?cohortid = RHEA) within the NewGeneris FP6 project, in which several biomarkers were measured in order to detect correlations between a maternal diet and genome damage in newborns.

The aim of our study was to assess the relationship between birth weight, gestational age and genome damage in newborns. Furthermore, it was examined if the NDI is associated with MNBN and MNMONO. Additionally, maternal and newborn NDI, MNBN and MNMONO are correlated.

2. Subjects and methods

The subjects analyzed in the present study are part of the Rhea mother child cohort of pregnant women in Heraklion, Greece. A detailed description of the total cohort is available in Vande Loock et al. [11]. A selected subgroup of this cohort consisting of 92 mothers and their newborns was analyzed. From the total Creta cohort, the best cultures were selected out, with the largest number of cells per subject allowing statistically meaningful fluorescent in situ hybridysation analysis knowing that micronucleus frequencies are relatively low. According to the age of mothers, their smoking habit, newborn gestational age, birth weight and gender subgroup is similar to the main cohort.

The study was performed within the 6th European Union Framework Program New Generis, which investigated the impact of the maternal diet during pregnancy on the increased risk of cancer development in children using different biomarkers. Briefly stated, a detailed questionnaire was used for collecting information on the mothers' types of exposure, habits and diets. Gestational age (GA) was determined by the interval between the last menstrual period and the date of delivery. In case when such an estimation of gestational age was not in concordance with the ultrasound examination taken in the first trimester of the pregnancy, a quadratic regression formula was used to describe the relationship between crown-rump length and GA as this method is shown to be the optimal in comparison with linear and cubic functions [19]. In the current study 48.9% of newborns were boys.

Maternal peripheral blood and cord blood were collected in heparinized tubes (BD Vacutainer, Plymouth, UK) immediately after delivery. An extra 0.5 mL of heparin (Leo Pharmaceutical Products, Ballerup, Denmark) was added into the tubes containing cord blood in order to prevent clotting [11]. The samples were kept at 4 °C and processed within 24 h.

The micronucleus assay was performed using whole-blood cultures cultivated at 37 °C. Umbilical cord blood was diluted (1:3) with phosphate buffer saline before culture preparation. Phytohaemagglutinin (Remel, Kent, UK) was used as a stimulator of proliferation and after 44 h, cytochalasin B (Sigma, Steinheim, Germany) was added to the final concentration of 6 μg/mL. After 72 h, the cell cultures were harvested and transferred to a cold hypotonic treatment using 90 mM KCl (Fisher Bioreagents, Pittsburgh, PA, USA) for umbilical cord blood and 110 mM KCl for maternal venous blood. After fixation, the cells were dropped on slides and stained for 20 min by Giemsa in a Sorensen buffer (pH 8.8; Prosan, Merelbeke, Belgium). The slides were prepared at the University of Crete, School of Medicine, using an Olympus light microscope (Olympus Optical, London, UK).

For each subject, 2000 binucleated and 2000 mononucleated cells were manually scored using the criteria of the Human MicroNucleus project (HUMN) [20] by one scorer. Also, NPB and NB were scored by the same scorer and the nuclear division rate calculated by the following formula:

$$\mathrm{NDI} = \frac{\text{No. of mononuleated cells} + 2 \times \text{No. of binucleated cells} + 3 \times \text{No. of multinucleated cells}}{\text{total No. of cells}}$$

2.1. C-FISH study

Fluorescent in situ hybridization was used for the scoring of centromere positive MN (C-FISH), which was performed in a subgroup of 28 pairs of mothers and their newborns with highest MN frequency. For each group, 1000 binucleated cells per individual were scored.

FISH was performed using centromeric probes produced by Cytocell Technologies Ltd., (Cambridge, UK) and directly labeled with red fluorophore (Texas Red spectrum), according to standard protocol and counterstained by10 μl of DAPI (4,6-diamino-2-fenilindol). Slides were viewed under an Olympus AX70 fluorescent microscope (Olympus Optical, London, UK). Image processing exposure was carried out using the CytoVision® FISH computer program (Applied Imaging, Germany).

2.2. Statistical analysis

Statistical analysis was performed using STATISTICA, version 7.1 (StatSoft, Inc., Tulsa, OK, USA). Basic descriptive summaries of the data were obtained using mean and standard deviation (SD). The differences between newborn children and their mothers were calculated using a *T*-test for dependent samples or the Wilcoxon matched pairs test, depending on the distribution of variables. Subgroups were defined according to whether the mother smoked during pregnancy ("yes" vs. "no"), low birth weight (<2500 g vs. ≥2500 g), and the frequency of MN in MO (<2 vs. ≥2) per 1000. Differences between subgroups were calculated using a Student's *t*-test or Mann–Whitney *U*-test, depending on the distribution of variables, Spearman rank order correlations were used for associating variables. The multivariate linear/nonlinear all effects model regression analysis was done using newborns' biomarkers such as dependent and gestational age, birth weight, mothers' smoking and mothers' biomarkers as independent variables for the models, along with the inclusion of all independent variables. Goodness