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Polychlorinated biphenyls (PCBs) alter DNA methylation and genomic integrity of sheep fetal cells in a simplified *in vitro* model of pregnancy exposure☆

Debora A. Anzalone[a], Silvestre Sampino[a,b], Marta Czernik[a], Domenico Iuso[a], Grazyna E. Ptak[a,b,c,*]

[a] Department of Biomedical Sciences, University of Teramo, Italy
[b] Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Poland
[c] Malopolska Centre of Biotechnology, Jagiellonian University, Cracow, Poland

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ABSTRACT

Polychlorinated biphenyls (PCBs) are persistent organic pollutants ubiquitously detectable in the environment and in the food chain. Prenatal exposure to PCBs negatively affects fetal development and produces long-term detrimental effects on child health. The present study sought to evaluate the cytotoxic and genotoxic effects of chronic PCB exposure on fetal cells during pregnancy. To this aim, sheep embryonic fibroblasts (SEF) and amniocytes (SA) were cultured *in vitro* in the presence of low doses of PCBs for a period of 120 days, comparable to the full term of ovine pregnancy. Cellular proliferation rates, global DNA methylation, chromosome integrity, and markers of DNA damage were evaluated at different time points. Moreover, SEF treated with PCBs for 60 days were left untreated for one further month and then examined in order to evaluate the reversibility of PCB-induced epigenetic defects. PCB-treated SEF were more sensitive than SA treated with PCBs, in terms of low cell proliferation, and increased DNA damage and global DNA methylation, which were still detectable after interruption of PCB treatment. These data indicate that chronic exposure of fetal cells to PCBs causes permanent genomic and epigenetic instability, which may influence both prenatal and post-natal growth up to adulthood. Our *in vitro* model offer a simple and controlled means of studying the effects of different contaminants on fetal cells - one that could set the stage for targeted *in vivo* studies.

1. Introduction

Polychlorinated biphenyls (PCBs) are organic chlorine compounds that were largely used as lubricating oils in transformers and capacitors, and as hydraulic fluids, plasticizers, and pesticides (Safe, 1992) until were found highly toxic and banned in the 1970s. Despite the ban, PCBs are still persistent and widely dispersed in the environment and in the food chain (La Rocca and Mantovani, 2006; Schecter et al., 2010). Being highly lipophilic and chemically stable, PCBs undergo limited catabolism after absorption, accumulate in the liver and adipose tissues, and are easily transferred to the fetus through the placenta (Park et al., 2008; Grandjean et al., 2012); therefore, PCBs contamination during pregnancy could be considered as inheritable. In addition to the PCBs themselves, their metabolites can also cross the placenta and reach the developing fetus (Fängström et al., 2005; Soechitram et al., 2004; Berg et al., 2010; Grimm et al., 2015). Exposure to PCBs during intrauterine life has been associated, in humans and animals, with several health effects such as birth weight reduction, disruption of reproductive system development, immune dysfunction, and altered brain development (Schantz et al., 2003; Fowler et al., 2008; Fudvoye et al., 2014; Lignell et al., 2016; Kristensen et al., 2016).

There are 209 possible congeners of PCBs with different biological activities (ATSDR, 2004). There is evidence about the involvement of the Ah receptor (Poland and Knutson, 1982; Safe, 1994) as well as other receptors that support a role of PCBs as endocrine disruptors and interfering with calcium homeostatic mechanisms (see reviews by Kodavanti and Tilson, 1997; Tilson and Kodavanti, 1998).

The present study sought to evaluate the consequences of chronic PCBs treatment on fetal cells using an *in vitro* system to simulate exposure in pregnancy, thus overcoming the ethical problems connected

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* Corresponding author at: Malopolska Centre of Biotechnology, Jagiellonian University, Gronostajowa 7A str., 30-387 Krakow, Poland.
E-mail address: g.ptak@uj.edu.pl (G.E. Ptak).

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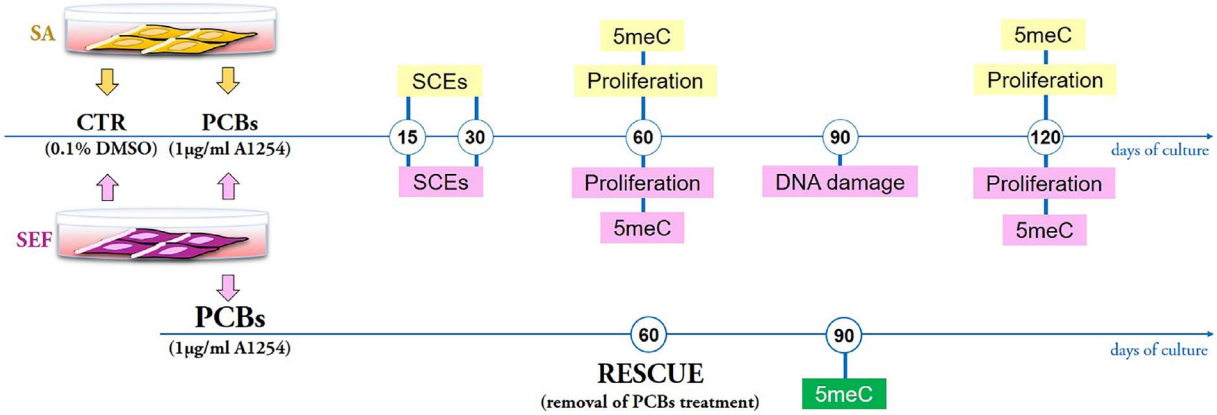

Fig. 1. *Experimental design.*
Primary culture of Sheep Amniocytes (SA) and Sheep Embryonic Fibroblasts (SEF) were obtained respectively from amniotic fluid and ear/paws of 1-month-old sheep fetus, previously recovered from the slaughterhouse. Once established, cell cultures were allocated in the following groups: *i*) PCBs group (1 μg/ml Aroclor1254); ii) control group (CTR) (0.1% DMSO) and *iii*) RESCUE group, namely SEF left untreated from 60th to 90th day of culture. Genetic and epigenetic damages were investigated at different time points, from 15th up to 120th day of culture: sister chromatid exchanges (*SCEs*), cell proliferation and global DNA methylation (*5meC*) were analyzed both in SA (*yellow*) and SEF (*pink*), while DNA damage (γ-*H2A.X histone* and *metaphases analysis*) was assessed only in SEF. To check reversibility of the effects, *5meC* was analyzed in RESCUE group (*green*) at 90th day of culture. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to *in vivo* treatment. A 120 day-old cultures of sheep fetal and amniotic cells were established to mimic long-term gestational exposure and to evaluate the sensitivity to PCBs exposure of embryonic *versus* extra-embryonic derived cytotypes. Data about genotoxic activity of PCBs mixtures are controversial, and little is known about the possibility that PCBs could interfere with epigenetic processes. Therefore, we evaluated global DNA methylation, chromosomal integrity and markers of DNA damage at different time points of PCBs exposure, as well as after interruption of the treatment, in order to evaluate the reversibility of PCBs-induced defects.

2. Materials and methods

All chemicals were purchased by Sigma-Aldrich, unless otherwise stated.

2.1. Experimental design

The experimental design is explained in Fig. 1. Starting from one month-old sheep *conceptus* (referred to as *fetus*) we have set up primary cell cultures, namely Sheep Embryonic Fibroblasts (SEF) and Sheep Amniocytes (SA), representative of fetal and extra-fetal compartments respectively. Cells were then allocated to control (CTR) and treated group (PCBs) up to 120 days of culture to mimic the entire duration of ovine pregnancy, and were analyzed at different time-points for Sister Chromatid Exchanges (SCEs), cell proliferation, and DNA global methylation. In proper fetal-derived cells, we also investigated DNA damage as γ-H2A.X histone foci and chromosomal abnormalities. Later, we tested whether the epigenetic damage, induced by PCBs, could be restores by short suspension of the treatment (RESCUE).

2.2. Cell culture

Cell lines used for the following experiments, have been derived from one month-old sheep fetus, previously collected from the local slaughterhouse as refuse animal material. Its age was determined based on the methods previously used (Ptak et al., 2013). Primary cultures of SEF were obtained from small pieces of fetal ears and paws by mechanical (by blades) and enzymatic (by trypsin digestion at 38.5 °C) disaggregation, while SA were isolated by centrifugation of amniotic fluid from the same fetus, 20 min at 1500 rpm. Cell lines were expanded in Minimum Essential Medium (MEM) enriched with 10% Fetal Bovine Serum (FBS), 2 mM L-Glutamine, 26 mM $NaHCO_3$ and 50 μg/ml Gentamicin. After three passages, cells were assigned to the following groups: *i*) treated group (PCBs), cultured in MEM with 1 μg/ml Aroclor1254 (A1254); *ii*) control group (CTR), cultured in MEM enriched with 0.1% DMSO (vehicle of PCBs); *iii*) RESCUE group, where PCBs treatment was suspended from 60th to 90th day of culture. Medium was replaced every 48 h and cells were passaged whenever 80% of confluency was reached. As for our previous work (Ptak et al., 2013), the working dose of contaminants tested in this study (1 μg/ml A1254) was chosen on the basis of PCBs concentration found in reproductive tissues and plasma from women of reproductive age.

2.2.1. Cell proliferation assay

Cell proliferation analyses was evaluated by an immunocytochemistry assay for 5-bromo-2′-deoxyuridine (BrdU), a thymidine analog incorporated during S-phase in replicating cells if previously added to culture medium. One day before the immunocytochemistry, cells were plated in multiwell slides (Millicell EZ Slide, Millipore), in number of 10,000/well. Briefly, SEF and SA at 60th and 120th day of culture were incubated with 100 μM BrdU for 4–6 h, fixed in cold methanol for 20 min and permeabilized with 0.1% Triton X-100, 15 min at room temperature (RT). Next, cells were treated with 4 N HCl 30 min, RT, and incubated with primary antibody (Ab I) (*mouse anti-BrdU, monoclonal antibody, B2531, Sigma*) 1:100 in blocking solution (BS) (0.1% Bovine Serum Albumin (BSA) in PBS) at 4 °C, overnight. Thus, cells were incubated with secondary antibody (Ab II) (*rabbit anti-mouse IgG-FITC polyclonal antibody, F9137, Sigma*) 1:500 in BS for 2 h RT, and counterstained with 0.5 μg/ml *propidium iodide* (PI) for 5 min. Between all steps, cells were washed twice in PBS, 5 min, RT. Finally, slides were mounted with Fluoromount and observed under an epifluorescent microscope Nikon Eclipse E600, at 20 × magnification. All nuclei were counted, while only the BrdU-positive nuclei have been considered as replicating cells. Cell proliferation rate was obtained by the ratio between the number of BrdU-positive cells (green) and the total number of cells (red). For statistical valence, we considered at least 200 nuclei for each group.

2.2.2. Global DNA methylation

Global methylation of DNA was investigated by immunofluorescence for 5-methylcytidine (5meC), on SEF and SA at 60th and 120th day of culture and on RESCUE group at 90th day of culture. One day before the immunocytochemistry, cells were plated in multiwell slides (Millicell EZ Slide, Millipore), in number of 10,000/well. Briefly, cells were fixed in 4% paraformaldehyde, 15 min RT, permeabilized