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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☆

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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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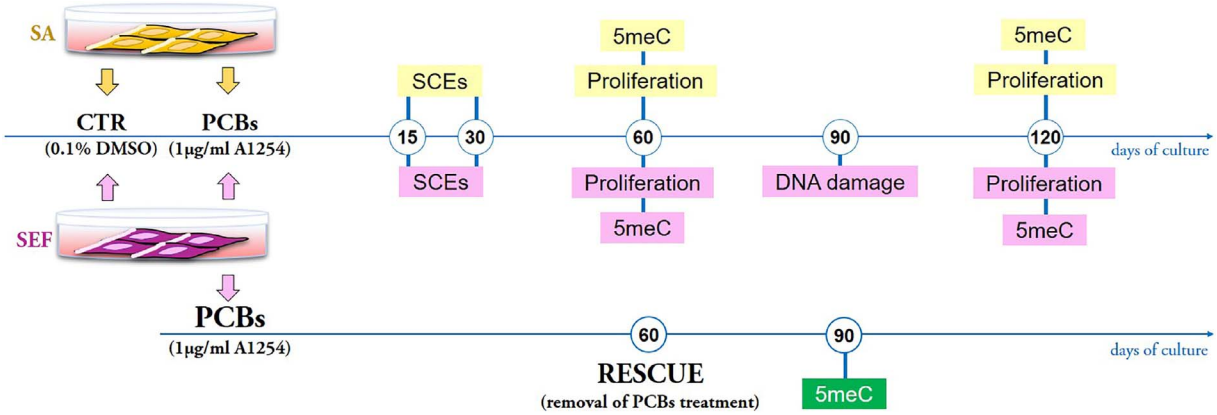

Fig. 1. *Experimental design.*
Primary culture of Sheep Amniocytes (SA) and Sheep Embryonic Fibroblasts (SEF) were obtained respectively from amniotic fluid and ear/paws of 1-month-old sheep fetus, previously recovered from the slaughterhouse. Once established, cell cultures were allocated in the following 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

with 0.1% Triton X-100, 15 min, and treated with 4 N HCl for 30 min RT. Then, cells were washed with 100 mM Tris/HCl, pH 8.5 for 15 min and incubated with Ab I (*mouse anti 5-methylcytidine, monoclonal antibody, 33D3, Santa Cruz Biotechnology*) 1:100 in BS, one hour, RT. Next, cells were incubated with Ab II (*rabbit anti-mouse IgG-FITC polyclonal antibody, F9137, Sigma*) 1:500 in BS, 2 h RT, and were counterstained with 0.5 μg/ml PI. Between passages, cells were washed twice in PBS for 5 min. Finally, slides were mounted with Fluoromount and observed under an epifluorescent microscope Nikon Eclipse E600, at 20 × magnification. DNA methylation level was quantified by analyzing the relative densitometry of nuclear fluorescence intensity, using Image Measurement and Analysis Lab software (IMAL 3.5.10.d). This software turns every fluorescent nucleus (methylation signals) into quantifiable data, subtracting the background noise. For statistical valence, we considered at least 200 nuclei for each group.

2.3. DNA damage assessment

2.3.1. Sister chromatid exchange (SCE)

Exchanges of genetic material between two sister chromatids represents recombinogenic events arising at DNA lesions. Sister chromatid exchanges can be detected following the incorporation of the thymidine analogue BrdU into DNA after two consecutive cell cycles. The SCEs are distinguishable after staining chromosomes with fluorescent dye, such as acridine orange (AO): the chromatids containing BrdU in both DNA strands, shows weaker fluorescence then those containing BrdU only in one, which appear more brightly. To visualize SCEs, we first incubated cells with 10 μg/ml BrdU for two consecutive S-phases of cell cycle (40 h for fibroblasts and 42 h for amniocytes). To obtain chromosomal metaphases, 0.5 μg/ml demecolcine was added to the culture medium 3 h prior to cell collection. Once trypsinized, cells were gently re-suspended in 10 ml of hypotonic solution (0.9% Na citrate/0.4% KCl in H_20, 1:1) and left for 20 min at 38.5 °C, after which 1 ml of fresh fixative (methanol/glacial acetic acid 3:1) was gently added to the solution. The suspension was centrifuged 5 min at 1200 rpm and the pellet resuspended in 10 ml of fresh fixative, 15 min at 4 °C. The solution was finally centrifuged for 5 min at 1200 rpm and the pellet resuspended in 1 ml of fresh fixative. Finally, 3/4 50 μl drops of the final suspension were spreaded on clean slides, stained with 2 mg/ml *acridine orange* (AO) and observed under an epifluorescent Nikon Eclipse E600 microscope at 40 × magnification. We counted a minimum of 50 metaphases to estimate the percentage of SCEs *per* cell.

2.3.2. Double-strand breaks (DSBs)

DSBs were evaluated by immunocytochemistry for γ-H2A.X phosphorylated histone, an early marker of DNA damage, in SEF at 60th and 120th day of culture. One day before the immunocytochemistry, cells were plated in multiwell slides (Millicell EZ Slide, Millipore), in number of 10,000/well. Cells were fixed and permeabilized as described above for BrdU assay, then incubated with Ab I (*mouse anti-phospho-Histone H2A.X, Ser139, clone JBW301, Millipore*) 1:100 in BS at RT for 2 h and with Ab II (*rabbit anti-mouse IgG-FITC polyclonal antibody, F9137, Sigma*) 1:200 in BS at RT for 2 h. Between passages, cells were washed twice in PBS for 5 min. Finally, cells were counterstained with 0.5 μg/ml PI, then slides were mounted with Fluoromount and observed under the epifluorescent microscope Nikon Eclipse E600. Notwithstanding an "intrinsic" phosphorylation of γ-H2A.X can occur during cell cycle progression (MacPhail et al., 2003), γ-H2A.X foci are smaller and less distinct than those induced by chemical agents (Huang et al., 2005). For this reason, only cells showing at least five distinct nuclear *foci* with high fluorescence intensity (*γ-H2A.X HFI foci*) were considered as injured cells. Damage rate was obtained by the ration between injured cells and total cell number. For statistical valence, at least 200 nuclei for each group were considered.

2.3.3. Cytogenetic analysis

Chromosomes were investigated in SEF at 60th and 90th day of culture. Metaphases were obtained as described above for the SCEs assay. Briefly, 0.5 μg/ml demecolcine was added to the culture medium 3 h prior to cell collection. Once trypsinized, cells were gently re-suspended in 10 ml of hypotonic solution (0.9% Na citrate/0.4% KCl in H20, 1:1) and left for 20 min at 38.5 °C, after which 1 ml of fresh fixative (methanol/glacial acetic acid 3:1) was gently added to the solution. The suspension was centrifuged 5 min at 1200 rpm and the pellet resuspended in 10 ml of fresh fixative and incubated 15 min at 4 °C. Finally, the solution was centrifuged for 5 min at 1200 rpm and the pellet was resuspended in 1 ml of fresh fixative. Once spreaded on clean slides, metaphases were stained in 8% Giemsa (in H_2O) and observed under the Nikon Eclipse E600 microscope. For each metaphase, we evaluated the gain/loss of chromosomes (numerical abnormalities) and structural defects - such as chromosomal fragments and the presence of extra metacentric chromosomes - more than six – (structural anomalies). Thus, we grouped abnormal cells as follows: with only numerical chromosomal aberrations (NCA), with only structural chromosomal aberrations (SCA) and with a combination of both numerical/structural chromosomal aberrations (N/S-CA). For statistical valence, we considered at least 50 metaphases were counted for each group.

2.3.4. Statistical analysis

Statistical significance was determined by GraphPad Prism 5.0 software. A *Chi-square test* was used for BrdU-proliferation assay, γ-H2A.X and cytogenetic analysis; a *t*-test (Mann-Whitney test) was used to evaluate global DNA methylation and to compare the frequency of SCEs among groups. We considered probability value $(p) < 0.05$ as statistically significant.

3. Results

3.1. PCB treatment alters proliferation

PCBs-treated SEF displayed low proliferation along the culture (at 60th and 120th day of culture) compared to the control group ($p < 0.05$; Fig. 2, SEF). On the other hand, proliferation of PCBs-treated SA decreased in a time-dependent manner, since there was a significant difference in the percentage of BrdU-positive cells between amniocytes exposed to PCBs for 60 days compared to those exposed for 120 days ($p < 0.05$; Fig. 2, SA).

3.2. PCBs induce permanent DNA hypermethylation in SEF

Treatment with A1254 resulted in a global DNA hypermethylation, detectable after 60 and 120 days of culture in SEF compared to control ($p < 0.0001$). Conversely, DNA methylation of SAs was not affected by A1254 treatment at any of the time points analyzed (Fig. 3). Due to the dynamic nature of epigenetic/processes in relation to changing environmental conditions, we hypothesized that the global DNA hypermethylation found in SEF following treatment could be rescued after removing PCBs from the medium for one month. Surprisingly, we found that SEF treated with PCBs for 60 days and then left untreated for one further month (totally 90 days of culture) still displayed an increase in DNA methylation comparable to SEF permanently treated (Fig. 3b, RESCUE).

3.3. PCB exposure induces mutagenesis

First, we evaluated the frequency of Sister Chromatid Exchanges *per* cell (SCEs/cell) in SEF and SA after 15 and 30 days of treatment, to determine the presence of recombinogenic events arising from DNA lesions. In both groups, we found higher SCEs rate in PCBs treated cells compared to control, at all analyzed time points ($p < 0.001$) as shown in Table 1 and fig. 4.

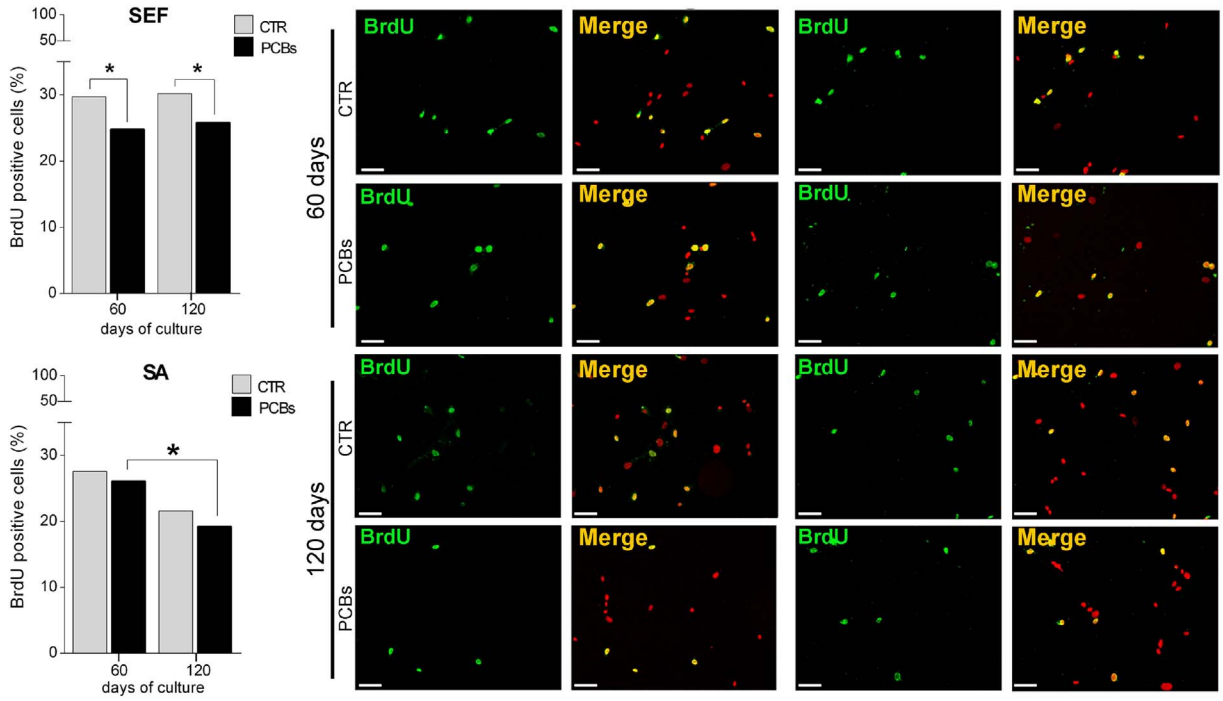

Fig. 2. *PCBs alter cell proliferation.*
The BrdU assay revealed that A1254 reduced cell proliferation in SEF (*a*) compared to control, at 60 and 120 days of culture. Amniocytes (SA) proliferation was not different between PCBs and CTR groups, but decreased in a time-dependent (*b*, 60 *vs* 120 days of culture). The images show BrdU positive cells (*green*) indicative of replicating cells. All nuclei are counterstaining with PI (*red*). Merge means PI + BrdU (*yellow*) (*$p < 0.05$). Scale bar = 20 μm; magnification is 20 ×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To assess the genetic status of the cells belonging to proper fetal compartments, we analyzed double-strand breaks and chromosomal metaphases in SEF. Immunofluorescence for the phosphorylated form of histone γ-H2A.X - marker of double-strand breaks – revealed an increase of γ-*H2A.X HFI* foci in PCBs-exposed fibroblasts than control, at 60th (28.9% *vs*. 21.3%, respectively) and 120th days of culture (44.5% *vs*. 34.4%, respectively) ($p < 0.05$; Fig. 5).

The examination of metaphases revealed that untreated cells of the control group carried more numerical aberrations then the PCBs-treated group, at all analyzed time points (60 days: 64.5% *vs*. 8.9%; 90 days: 51.4% *vs*. 6.9%, respectively; $p < 0.0001$) (Table 2, Fig. 6). Interestingly, PCBs-treated fibroblasts showed more serious damages, carrying both numerical and structural chromosomal aberrations, from the 60th (82.2% *vs*. 19.4%, $p < 0.0001$) to the 90th days of culture (87.9% *vs*.

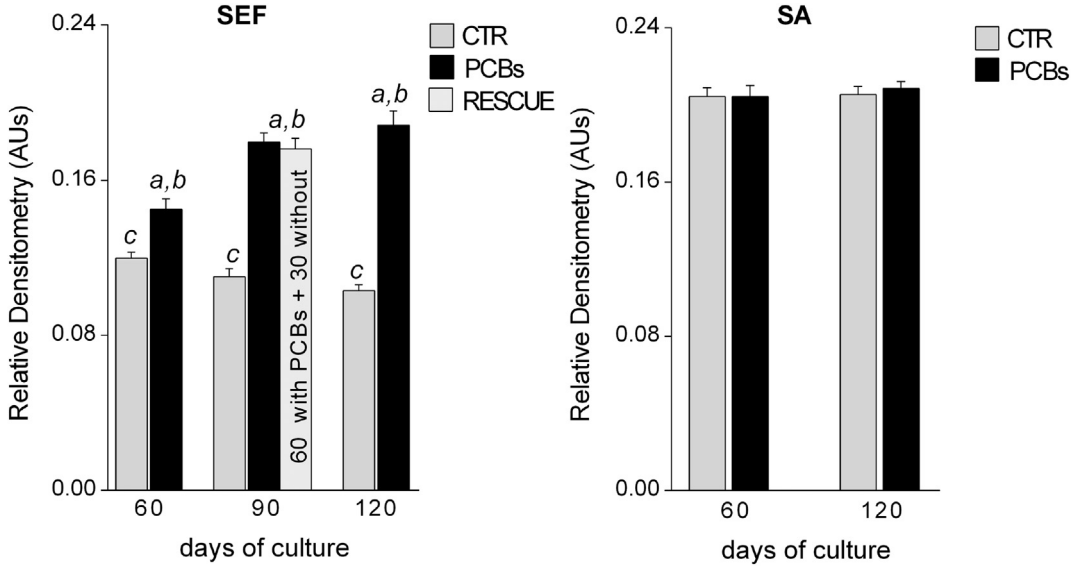

Fig. 3. *PCB treatment alters global methylation in SEFs but not in SAs.*
Global DNA methylation increased in PCBs treated fibroblasts (*left*, SEF) compared to control, at all analyzed time points and in a time dependent manner. An opposite trend was observed in control fibroblasts, where global DNA methylation progressively diminished during culture. The hypermethylation of DNA persisted after 30 days of treatment interruption (from 60th to 90th day of culture, RESCUE SEF). Conversely, global DNA methylation of sheep amniocytes (*right*, SA) was not affected by PCBs treatment nor by age of culture, since no differences were observed between groups at all time points. *a,b,c means $p < 0,0001$; respectively: PCBs/RESCUE vs CTR; PCBs 60 vs 90 vs 120; CTR 60 vs 90 vs 120.* Fluorescence intensity was quantified by relative densitometry and is given in arbitrary units (AUs).

Table 1
SCEs frequency in SEF and SA, at 15 and 30 days of culture.
The number of SCEs *per cell* was significantly higher both in fibroblasts (SEF) and in amniocytes (SA) exposed to PCBs compared to CTR, at 15 and 30 days. *a)* and *b)* mean $p < 0.0001$ PCBs *vs.* CTR in SEF and SA respectively.

Groups		Mean ± SD	
SEF	CTR	3.81 ± 2.13	4.50 ± 2.13
	PCBs	5.75 ± 2.33 [a]	7.46 ± 3.30 [a]
SA	CTR	4.37 ± 2.13	8.73 ± 3.59
	PCBs	6.97 ± 2.9 [b]	5.54 ± 2.14 [b]
Days of culture		15	30

32.4%, $p < 0.05$).

4. Discussion

Several epidemiological studies negatively correlate pregnancy exposure to polychlorinated biphenyls to impaired fetal development with growth and neurological problems in childhood. However, cellular mechanisms have not yet been clarified.

Here, we wanted to elucidate the cytotoxic effects of pregnancy exposure to PCBs, setting up a simple *in vitro* culture system that simulate the absorption of toxicants by anearly stage *conceptus*.

We found that PCBs exposure leads to recombinogenic events, double-strand breaks in the DNA and chromosomal aberrations, as well as epigenetic changes in the global DNA methylation that turn out to be permanent.

The presence of sister chromatid exchanges from the beginning of the culture is indicative of the rapid occurrence of DNA breakage (*i.e.* by homologous recombination) that cells attempts to repair.

Given that fibroblasts were more impaired than amniocytes in terms of cell proliferation and DNA methylation, and since amniocytes are transitory cells in the development while fibroblast are constituent part of the future organisms, we decided to investigate the DNA integrity of this latter cell type exclusively. It is known that phosphorylated form of γ-H2A.X histone represents an early marker of DNA damage, namely double-strand breaks (Bouquet et al., 2006; Löbrich et al., 2010). The high rate of γ-H2A.X foci on the DNA of PCBs-treated fibroblasts could represent the points of chromosomal rearrangement that lead to the DNA repairing event through homologous recombination by sister chromatid exchanges (Conrad et al., 2011). A strong relationship between PCBs exposure and elevated levels of SCEs and γ-H2A.X foci has been previously reported (Venkatesha et al., 2008). It is possible that this genotoxic damage, not completely repaired, lays the groundwork for the bulk of chromosomal aberrations, leading to aneuploidy and rearrangements (Varga and Aplan, 2005; Natarajan and Palitti, 2008). The presence of severe numerical and structural chromosomal abnormalities in PCBs-exposed SEFs confirms this scenario. Furthermore, among the bizarre anomalies, it was interesting to note the formation of new supernumerary metrocentric chromosomes, clearly smaller than normal ones, probably arising from the fusion/translocation of two small acrocentric chromosomes.

Adverse environmental conditions – such as exposure to cytotoxic agents – may also result in epigenetic changes (Laurent et al., 2010) likely associated with proliferative blocks, which can arise during intrauterine life and persist from birth to death (Guerrero-Preston et al., 2011). Of note, we observed that the hypermethylation induced by PCBs in fetal cells is not reversible by interrupting the exposure to A1254. This suggests that prenatal exposure to A1254 induces the development of a permanently altered epigenetic profile that might be maintained long-term. Chromosomal instability may occur in response to epigenetic changes (Herrera et al., 2008), because though aneuploidy reduces an organism's fitness (Torres et al., 2010), an abnormal karyotype could confer advantageous properties for cell physiology, by changing gene copy numbers and subsequently, the protein balance (Sheltzer and Amon, 2011; Yona et al., 2012). Altogether, these biological alterations may contribute to the development of an abnormal phenotype. Many evidences correlate the presence of PCBs into the biological fluids of pregnant women (Bloom et al., 2007; Givens et al., 2007; Meeker et al., 2009) to negative child health outcomes (Hertz-Picciotto et al., 2005; Grumetto et al., 2015; Ashley-Martin et al., 2015; Hansen et al., 2016). We observed that fetal fibroblasts and amniocytes responded differently to PCB exposure probably because of their different roles into the organism and their different biological features, such as lipid content. PCBs possess high lipophilic properties (Yu and Mylander, 2011) and can accumulate mainly in cells with high lipid content (Müllerová and Kopecký, 2007; Bourez et al., 2012). In this regard, the literature reveals a lower degree of PCBs accumulation in the amniotic fluids compared to other biological compartments, such as

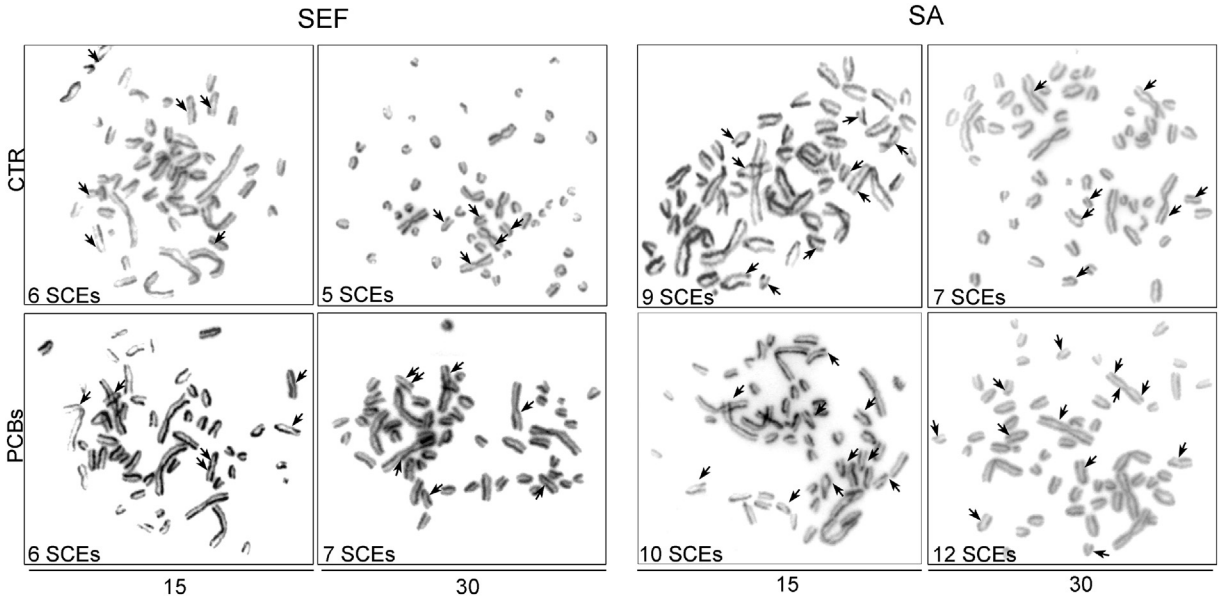

Fig. 4. *Increased frequency of Sister Chromatid Exchanges (SCEs) in fibroblasts and amniocytes exposed to PCBs.*
Images show sister chromatid exchange (SCEs) in metaphase chromosomes obtained from fibroblasts (SEF) and amniocytes (SA) at 15 and 30 days of culture; the exchanges of genetic material are detectable from colour difference of two chromatids and are indicated by arrows.

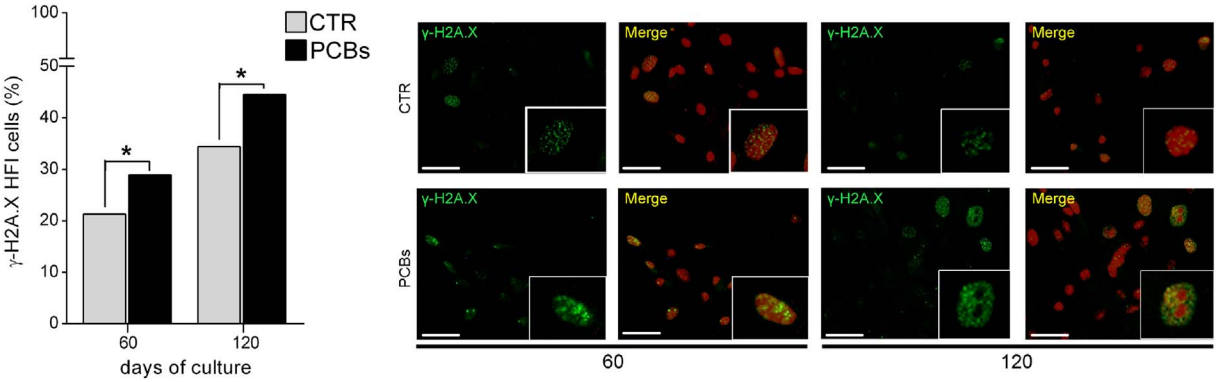

Fig. 5. *PCBs enhance γ-H2A.X phosphorylation, increasing DNA damage in treated fibroblasts.*
PCBs increased the percentage of fibroblasts with *γ-H2A.X HFI foci* at 60 and 120 days of culture (CTR *vs* PCBs, $*p < 0.05$). Images show *high-intensity foci* of γ-H2A.X (green spots) in CTR and PCBs-treated cells with details. All nuclei were counterstained with PI (red). Merge means PI + γ-H2A.X (*yellow*). Scale bar = 20 μm; magnification is 40 ×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Frequency of chromosomal abnormalities in SEF.
PCBs exposure induced the development of chromosomal abnormalities in SEF (90% *vs* 62%, PCBs *vs* CTR). Numerical (NCA), structural (SCA) and both types (NCA-SCA) of chromosomal abnormalities were calculated for each experimental group. NCA were more expressed in the control group ($p < 0.05$), while PCBs-exposed fibroblasts displayed a high number of both numerical and structural chromosomal abnormalities at 60 and 90 days of culture compared to the control group ($p < 0.0001$). *a, b)* mean respectively $p < 0.05$, $p < 0.001$ and $p < 0.0001$ between PCBs *vs.* CTR.

SEF	Abnormal cells (%)	*NCA* (%)	*SCA* (%)	*N/S-CA* (%)	Abnormal cells (%)	*NCA* (%)	*SCA* (%)	*N/S-CA* (%)
CTR	31/50 (62%)	20/31 (64.5%)	5/31 (16.1%)	6/31 (19.4%)	37/65 (56.9%)	19/37 (51.4%)	6/37 (16.2%)	12/37 (32.4%)
PCBs	45/50 (90%)[b]	4/45 (8.9%)[a]	4/45 (8.9%)	37/45 (82.2%)[c]	58/60 (96.6%)[a]	4/58 (6.9%)[a]	3/58 (5.2%)	51/58 (87.9%)[c]
Days of culture	60				90			

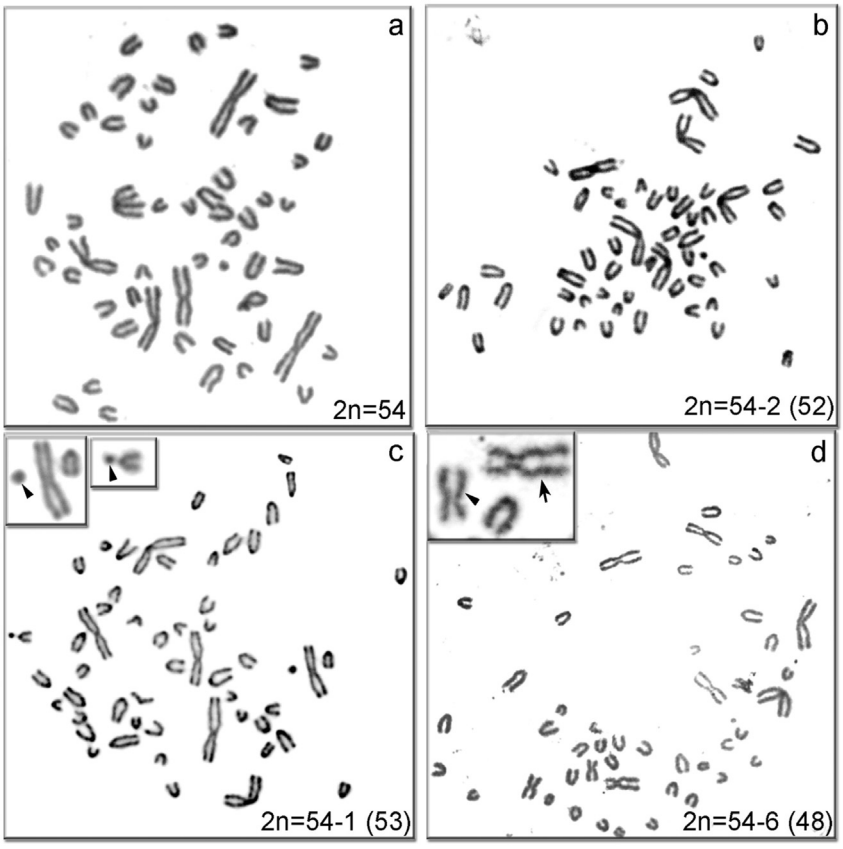

Fig. 6. Kinds of chromosomal abnormalities in PCB-treated fibroblasts.
Images show some representative metaphase chromosomes found in SEF: a) normal karyotype; b) aneuploidy, 2n-2 = 52; c) structural abnormalities (2n-1 = 53) (magnification shows little fragment of chromosome, not distinguishable from the Y chromosome, both indicated by arrowhead); d) numerical and structural abnormalities (magnification shows small micro-metrocentric-arrowhead-and submetrocentric-arrow- chromosomes, not detectable into a normal ovine karyotype).

the placenta (Foster et al., 2000; Jarrell et al., 2005). It is easier to discover some PCB metabolites than congeners (Gallenberg et al., 1990; Grimm et al., 2015). This observation invites us to reflect about the analysis of extra-embryonic cells (*i.e.* amniocytes) in women exposed to PCBs during gestation, because could underestimate the level of PCBs, providing values not correspondent to the real rate of contamination within the proper fetal compartments. These very serious and irreversible genetic and epigenetic abnormalities may influence intrauterine life, compromising both prenatal and post-natal growth up to adulthood.

This *in vitro* system - which is similar to gestation for cells and duration of culture - fills important gaps in the knowledge of how pregnancy exposure to PCBs causes genotoxic damage, and sets the stage for targeted *in vivo* studies.

Conflict of interest

The authors have no competing interests to declare.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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