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EFFECTS OF NON DIOXIN LIKE POLYCHLORINATED BIPHENYLS IN 3D4/31 MACROPHAGE CELL LINE

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Summary

Polychlorinated biphenyls (PCBs) are persistent organic pollutants (POPs) causing adverse effects on both humans and animals such as endocrine disruption, neuro and immunotoxicity. Immune system is an important target organ for many environmental contaminants. Macrophages are important components of the immune system of both humans and animals; in fact, they are responsible for triggering innate immune response and host defence. Although it is well known that different environmental pollutants may affect immune functions, only few studies have been carried out to date about the effects of PCBs in macrophages; while, at our knowledge, no study investigated the effects of these contaminants in porcine macrophages. The aim of the current study was to evaluate the effects of three non dioxin like PCB congeners (PCB 138, PCB 153 and PCB 180) alone and differently combined, on porcine macrophages cell line (3D4/31). The results of the current study showed that ndl-PCBs reduced significantly cell viability only at the highest tested concentrations (50 µM); such effect was not linked to apoptosis induction or cell cycle arrest. The contemporary presence of more than one contaminant (differently combined) did not induce any enhancement of effects on 3D4/31 cell line. These data highlighted the need to continue the evaluation of toxic properties of ndl-PCBs, which represent less studied PBCs, in particular in term of risk assessment.

Keywords: Polychlorinated biphenyls; porcine macrophages; food contaminants

Introduction

Polychlorinated biphenyls (PCBs) are persistent organic pollutants (POPs) recognized as dangerous both for humans and animals as well as the ecosystem (Stockholm Convention on Persistent Organic Pollutants, 2001).

PCBs are 209 different congeners, divided in two groups: 12 dioxin-like PCBs (non-ortho substituted PCBs) and 197 non dioxin-like PCBs (ortho or mono-ortho substituted PCBs). These contaminants have been widely produced by industry until 1980s because of their dielectric and flame retardants properties. Nowadays, production, commercialization and use of PCBs are prohibited within the European Union (1).

Humans and animals are exposed to these contaminants for 95% trough the consumption of contaminated food and feed (2). Maximum tolerable levels of dl-PCB and TCDD/Fs have been set by European Regulation (3) in different types of food. Recently, maximum tolerable levels in food have been set also for ndl-PCBs (4).

Exposure is usually associated with adverse effects in both humans and animals. These effects are commonly attributed to dl-PCBs, although ndl-PCBs were demonstrated to act on neuronal cells causing the reduction of dopamine neurotransmitter levels or the interference with calcium homeostasis (5), tumor promotive activity in mice (6) and co-carcinogenic effects in rats (7, 8) and to have effects on signalling pathways contributing to liver tumor promotion (9) and inhibition of apoptosis (10).

Several toxic effects on the immune system have been ascribed to PCBs. In vitro and in vivo studies have shown that PCB are immunotoxic chemicals, in particular, they exert the action on cell-mediated immunity (11), inhibition of lymphocyte blastogenesis in animal species (12), altered expression of cytokines (13). PCBs seem to be able to modify macrophage function. Macrophages are important component of the immune system of humans and animals, in fact, they are responsible for triggering innate immune response of host defense and engulfing microorganisms, particularly bacteria (14). Moreover, they play an important role for the clearance of apoptotic cells and the tumor immunosurveillance (15, 16) and in induction and maintenance of the inflammatory process by the expression of different cytokines as well as producing nitric oxide (NO), with bactericidal action, which is involved in many physiological processes (17).

In spite of extensive documentation of the toxic effects of PCBs on both human and animal health, a complete and detailed explanation of the mechanisms by which these contaminants exert toxic effects on macrophages is not yet been completely showed. At the moment, only few studies have been carried out to evaluate the effects of these food contaminants on macrophages (18); at our knowledge, no study investigated the effects of ndl-PCBs on porcine macrophages. Moreover, no data are available on the effects of their mixture on macrophage activity.

Therefore, the aim of the current study was to assess the immunomodulatory effects of most common congeners of ndl-PCB by using porcine macrophage cell line (3D4/31). We chose these congeners because they are consider to be indicator of ndl-PCB contamination in food. A survey of food and feed carried out by European Food Safety Authority (2) reported that six most found PCBs congeners in food products were ndl-PCBs, in particular PCB 28, 52, 101, 138, 153 and 180, accounting for more than 50% of all congeners present in foodstuffs of animal origin and in human fat. Moreover, they are included in the Italian Official Control Plan on food related to detection of drugs and contaminants residues. European laws have recently fixed maximum tolerable limit for these contaminants in different food and food products (4).

We evaluated not only the effects induced by the individual congeners but also those deriving from mixtures of these contaminants in consideration that co-contamination of the same food substrate is common in natural situation and the effects of

simultaneous exposure to multiple xenobiotics are still little investigated.

Methods

Reagents

Unless stated otherwise, all reagents and compounds were obtained from Sigma Chemicals Company (Sigma, Milan, Italy).

PCB 138 (2,2',3,4,4',5'-Hexachlorbiphenyl; CAS No 35065-28-2; Fluka), PCB 153 (2,2',4,4',5,5'-Hexachlorbiphenyl; CAS No. 35065-27-1; Fluka), PCB 180 (2,2',4,4',5,5'- Hexachlorbiphenyl; CAS No. 35065-29-3; Fluka), were chosen for the current study because they are the congeners most commonly found in food (2).

Cell Culture

3D4/31 cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1% NEAA, 1% Na piruvate and antibiotics (PEN 100 U/ml; STREP 100 µg/ml) at 37 °C in humidified atmosphere with 5% CO_2.

Cytotoxicity assay

Cells (5 x 10^3 cells/well) were plated on 96-well microtiter plates and allowed to adhere. Thereafter medium was replaced with serial dilutions of PCB 180, 153, 138 in fresh medium. Ndl-PCBs were dissolved in DMSO and then in culture medium to obtain final concentrations ranging from 1 to 50 µM). Each compound was evaluated both alone or in combination for further 24, 48 and 72 h, and cell viability was then assessed through MTT assay according to an established protocol (19). Briefly, 25µL of MTT (5 mg/mL) were added and the cells were incubated for 3 h. Thereafter, cells were lysed and the dark blue crystals solubilised with 100µL of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. 3D4/31 cell viability in response to treatment with PCBs was calculated as: % cells=(OD treated/OD control) x 100.

Analysis of apoptosis

Hypodiploid DNA was analyzed using PI staining by flow cytometry. Briefly, 3D4/31 cells (3.5 x 10^5 cells/well) were grown in 24-well dishes and allowed to adhere. Then, cells were incubated with PCB (1-50 µM) for further 24, 48 and 72 h. Following the treatment, culture medium was removed, cells washed once with PBS and then resuspended in 500 µL of a solution containing 0.1% (w/v) sodium citrate, 0.1% Triton X-100 and 50 µg/mL propidium iodide (PI).

Culture medium and PBS were centrifuged and cell pellets were pooled with cell suspension to retain all dead and living cells for analysis. After incubation at 4 °C for 30 min in the dark, cell nuclei were analyzed with Becton Dickinson FACScan flow cytometer using CellQuest program and the DNA content of the nuclei was registered on a logarithmic scale. Cellular debris was excluded from the analysis by raising the forward scatter threshold and the percentage of cells in the hypodiploid region (sub G_0/G_1) was calculated.

Analysis of cell cycle

In order to determine the effect of ndl-PCBs on cell cycle we tested each compound, alone and in combination, at different concentrations (1-25 µM). These concentrations were chosen because they did not induce significant cytotoxicity. 3D4/31 cells were seeded in a 12-well plastic plate (7.0 x 10^5 cells/well) and allowed to adhere; therefore cells were treated with compounds for 24, 48 and 72 h. After incubation, 3D4/31 cells were harvested and fixed in cold 70% ethanol at -20 °C. Cell cycle profiles were evaluated by DNA staining with PI (2.5 mg/ml) in

phosphate-buffered saline (PBS) supplemented with 100 U/mL ribonucleases A, for 30 min at room temperature. Samples were analysed with a FACScan flow cytometer (Becton Dickinson, CA) using Mod FitLT program.

Statistical analysis

All the values shown in tables, figures and text are expressed as mean standard deviation (SD) of at least three independent experiments. A P-value less than 0.05 was considered as significant.

Results

Effects of ndl-PCBs on 3D4/31 macrophage viability

After incubation of 3D4/31 cells with graded concentrations of ndl-PCBs (1-50 µM), alone and in combination, for 24, 48 and 72 h, cell viability was determined by MTT assay. Control cells viability was designated as 100%. PCB 138, PCB 153, PCB 180 reduced significantly cell viability only at the highest concentrations (50 µM). The combination of three ndl-PCBs did not induce any increased effect on cell viability (Fig.1).

Effects of ndl-PCBs on apoptosis in 3D4/31 macrophages

Cytofluorimetric analysis of PI staining of hypodiploid nuclei was assessed incubating 3D4/31 macrophages with graded concentration of each PCB (1-50 µM), alone and in combination, for 24, 48 and 72 h. Our results indicate that none of examined compound significantly induced apoptosis in 3D4/31 cells, neither singly nor in mixture (data not showed).

Effects of ndl-PCBs on cell cycle progression in 3D4/31 macrophages

In order to determine if ndl-PCBs affect cell cycle cells distribution, we carried out a cytofluorimetric cell cycle analysis. We treated 3D4/31 with 1-25 µM of each ndl-PCBs, alone and in combination, for 24, 48 and 72 h. Exposure of 3D4/31 cells to each assessed environmental contaminant, alone and in combination, for 24, 48 and 72 h did not resulted in a significant alteration of the G_0/G_1, S and G_2 phase cell cycle distribution respect to untreated cells (data not showed).

Discussion

PCBs are environmental contaminants that can be found in many foods, primarily those of animal origin and derived products (2). Food and feed commodities are often contaminated by more than one pollutant (20) so humans and animals can be exposed simultaneously to different xenobiotics. Co-exposure to more than one contaminant represents a great concern for public health, particularly regarding the safety of consumers which can be exposed simultaneously to several xenobiotics through the consumption of different foods. Regarding infants, breast milk could represent another way of assumption of PCBs (21).

Moreover, carry-over rate of PCBs in goat milk ranged from 40 to 60% (22). Considering this, studying the interactions among different food contaminants can be very useful. Our previous studies about combined effects of different mycotoxins which are natural toxic food contaminants, reported no additive or synergistic effects of trichothecenes nivalenol and deoxynivalenol on murine macrophages (23) while aflatoxins (24) and some *Fusarium* mycotoxins could exert additive and synergistic effects on different immune-competent cells (25, 26, 27).

Following the ingestion, PCBs may exert their toxicity representing health risk both for humans and animals, affecting immunereactivity. In particular, macrophages play a central role in the immune response and are considered as the first line of defense against infectious agents.

The aim of the current study was to evaluate the immunomodulatory effects of three ndl-PCBs (138,

153 and 180) on macrophages functions by using 3D4/31 cells as macrophage model of pig.

Our results showed that ndl-PCBs 138, 153 and 180 exerted a significant cytotoxicity on 3D4/31 macrophages only at the highest concentration (50 µM) and no increased effects were observed by mixture of three ndl-PCBs indicating that their simultaneous presence did not enhance each own singular effect in this particular cell model.

In order to investigate the effects of ndl-PCBs 138, 153 and 180 on apoptosis induction we measured hypodiploid nuclei by cytofluorimetric analysis of PI staining. Our results indicate that none of examined compound significantly induced apoptosis in 3D4/31 cells, neither singly nor in mixture. Our results about ndl-PCBs ability to induce apoptosis agree with those obtained by Tharappel et al. (7) which found that PCB 153 inhibited apoptosis in rat hepatocytes. Nevertheless, our results are in disagreement with previous studies, which demonstrated that mixture of PCBs induced an antiproliferative effect in MCF-7 breast cancer cells, ascribable to an apoptotic action (28). In particular, Gosh et al. (29) showed that non-coplanar PCB 153 induced enhanced apoptotic death in human liver HepG2 cells and human kidney HK2 cells, although kidney cells are more vulnerable to PCBs. Another study revealed that PCB 153 can induce apoptosis in primary rat renal tubular cells likely through the PKC alpha, caspase-3 and Bcl-2/Bax pathway (30). Such discrepancies could be explain considering the different cellular models.

Analysis of cell cycle kinetic gave another insight into the mechanism of the effects of these contaminants on the 3D4/31 cells. Particularly, we also observed that none of the assessed environmental contaminant, alone and in combination, resulted in a significant alteration of the G_o/G_1, S and G_2 phase cell cycle distribution respect to untreated cells after 24, 48 and 72 h of exposure. On the contrary, significant difference in cell cycle phase distribution was observed in MCF-10A non-tumorigenic human mammary epithelial after 24 h of exposure to PCB 153; such effect was likely due to a decreased cyclin D1 expression (31).

In conclusion, the results of the current study highlight the need to continue the evaluation of toxic properties of ndl-PCBs, which are less studied PCBs; such studies could help us to better understand the molecular mechanism of their toxicity and provide useful information particularly in term of food safety and risk assessment.

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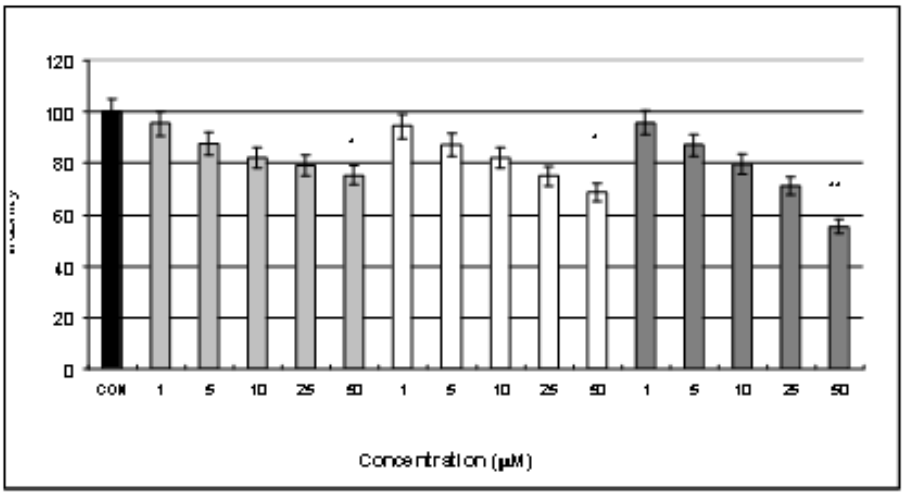
Figure legend

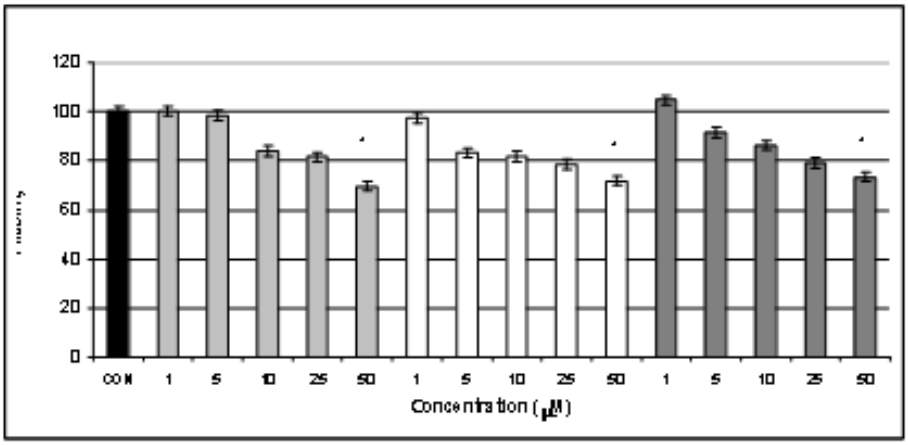
Fig. 1. Viability of 3D4/31 cells following the incubation with PCB 138, 153 and 180 (1-50 µM), alone and differently combined, for 24 (A), 48 (B) and 72 (C) h. Data are expressed as mean ± SD from at least three-independent experiments. *P<0.05; **P<0.01

Black: control;
light grey: PCB 180;
white: PCB 153;
dark grey: PCB 138.

A



B



C