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Original article

Intestinal absorption of ^{14}C from ^{14}C-phenanthrene, ^{14}C-benzo[a]pyrene and ^{14}C-tetrachlorodibenzo-*para*-dioxin: approaches with the Caco-2 cell line and with portal absorption measurements in growing pigs

Séverine CAVRET, Claire LAURENT, Cyril FEIDT, François LAURENT, Guido RYCHEN*

Laboratoire de Sciences Animales, INPL-UHP-INRA, École Nationale Supérieure d'Agronomie et des Industries Alimentaires, BP 172, 54505 Vandoeuvre-lès-Nancy, France

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Abstract — The aim of this work was to study the transfer through the intestinal barrier of two polycyclic aromatic hydrocarbons (PAHs) (benzo[a]pyrene and phenanthrene) and a dioxin (2,3,7,8-tetrachlorodibenzo-*para*-dioxin) which differed in their physicochemical properties. Both in vitro and in vivo assays were performed. For the in vitro study, Caco-2 cells, cultivated on permeable filters, permitted to measure the transepithelial permeability of the studied ^{14}C-labelled molecules. For the in vivo study, portal absorption kinetics were evaluated in pigs fed contamined milk. The results showed that all the molecules were absorbed and demonstrated a differential intestinal absorption for the studied molecules. Phenanthrene appeared to be the fastest and most uptaken compound, followed by benzo[a]pyrene and finally 2,3,7,8-tetrachlorodibenzo-*para*-dioxin. Their absorption levels were respectively 9.5, 5.2 and 1.4% after a 6 h-exposure in vitro and 86.1, 30.5 and 8.3% in vivo for the 24 h following ingestion. These findings suggest that the physicochemical properties of the xenobiotics and intestinal epithelium play key roles in the selective permeability and in the bioavailability of the tested micropollutants.

intestinal transfer / PAHs / dioxin / Caco-2 / portal absorption

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) and dioxins are ubiquitous organic molecules released in appreciable amounts in the environment [1–3]. The diet is one of the main sources of human and animal background exposure to these lipophilic pollutants [4, 5]. Food-animal transfer of these compounds plays a key role in determining

* Correspondence and reprints
E-mail: Guido.Rychen@ensaia.inpl-nancy.fr

Table I. Physicochemical properties of the studied organic micropollutants.

Compound	Fused benzene rings number	Lipophilicity Log K_{ow}	Water solubility ($mg \cdot L^{-1}$)	Molecular weight ($g \cdot mol^{-1}$)
2,3,7,8-TCDD	2	6.80	1.93×10^{-5}	312.98
Benzo[a]pyrene	5	6.31	3.80×10^{-3}	252.00
Phenanthrene	3	4.50	1.20	178.20

how the dietary exposure effectively translates into the tissue.

Little is known about the factors governing the intestinal absorption of these molecules in humans or animals. Many in vivo studies on dioxins and some works on PAHs report that the absorption mechanisms of these molecules depend on their matrices and their physicochemical properties (water solubility, lipophilicity and molecular weight mainly) [6–12]. Among PAHs, some studies with fish or rats suggest that the compounds are absorbed differently by the gastrointestinal tract, maybe as a function of their physicochemical characteristics [13, 14].

In the present study, we used two complementary animal models to specify the intestinal absorption of ^{14}C-labelled 2,3,7,8 tetrachlorodibenzo-*para*-dioxin (2,3,7,8-TCDD), benzo[a]pyrene and phenanthrene. At first, an in vitro model of the intestinal epithelial cells (the Caco-2 cell line), allowed to evaluate the uptake and its mechanisms at the intestinal level. Then, an in vivo model of a growing pig fitted with permanent catheters in the portal vein and the brachiocephalic artery provided an interesting model for portal absorption studies in humans [15, 16].

2. MATERIAL AND METHODS

2.1. Organic micropollutants

The studied micropollutants were chosen according to their different physicochemical properties (Tab. I). The difference between 2,3,7,8-TCDD and benzo[a]pyrene is mainly the fused benzene rings number whereas the difference between 2,3,7,8-TCDD and phenanthrene is the lipophilicity (or water solubility). The two PAHs have different lipophilicities and fused benzene rings number.

2.2. The in vitro model: Caco-2 cell line

Recently, the cultures of the Caco-2 cell monolayers, isolated from a human colon carcinoma, have been used as a valuable in vitro model to study the intestinal uptake and transport processes of hydrophobic xenobiotics such as polychlorinated biphenyls [17, 18]. Differentiated post-confluent Caco-2 cells exhibit well-developed microvilli and, when grown on semi-permeable supports, they form tight monolayers with a polarized distribution of brush border enzymes [19].

2.2.1. Cell culture

The basal Defined Medium (BDM) was composed of Iscove Modified Dulbecco Medium, F 12 Nutrient HAM, NCTC-135 medium (5/5/1) purchased by Invitrogen (Cergy-Pontoise, France) and glutamine (6 mM), sodium bicarbonate ($4\ g \cdot L^{-1}$) and ethanolamine (50 mM). In this medium, antibiotics, hydrocortisone, epidermal growth factor (EGF), triiodothyrosine, insulin, linoleic acid albumin, dimethylsulfoxide (DMSO) and phosphate-buffered saline (PBS) came from Sigma Aldrich (Saint

Quentin Fallavier, France). The fetal calf serum (FCS) was from Biowest (Nuaille, France). The collagene solution was from Cellon (Luxemburg), the trypsin (2.5 $g \cdot L^{-1}$)-EDTA (1.1 $g \cdot L^{-1}$) solution was from Biowhittaker (Emerainville, France), and the non-essential amino acids were from Invitrogen (Cergy-Pontoise, France). The cell culture inserts were supplied by Merck Eurolab (Fontenay-sous-Bois, France). The Caco-2 cells were obtained from the Cellular Biochemistry Laboratory (Louvain-la-Neuve University, Belgium). The cells from passage 210 to 225 were used in the experiments.

Caco-2 cells were routinely frozen in a culture medium containing 20% FCS and 10% DMSO. The cell line was seeded on filter inserts (4.2 cm^2, 1 μm pore size) at a cell density of about 160 000 cells·cm^{-2} and was maintained in BDM containing 1% non essential amino acids, insulin (1 $\mu g \cdot mL^{-1}$), hydrocortisone (0.1 μM), EGF (1 $ng \cdot mL^{-1}$), triiodothyrosine (2 nM), insulin (1 $\mu g \cdot mL^{-1}$), linoleic acid albumin (10 $\mu g \cdot mL^{-1}$), penicillin (100 $U \cdot mL^{-1}$ medium), streptomycin (100 $\mu g \cdot mL^{-1}$) and gentamycin (100 $\mu g \cdot mL^{-1}$), at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. The medium was changed every 2 days for 17 days.

Before transport study assessment, the integrity of the cell monolayers was first assessed by measuring the transepithelial electric resistance with a Mitchell-ERS Epithelia Voltohmmeter (Millipore Co., Bedford, USA). The monolayers of the cells showed a mean transepithelial resistance of 692 (sd = 21, n = 48) Ohms·cm^2. These resistances verified the epithelia integrity [20, 21].

2.2.2. Preparation of PAHs and 2,3,7,8-TCDD containing media

U-^{14}C-2,3,7,8-TCDD (1679.8 $MBq \cdot mmol^{-1}$, purity > 98%) was purchased from Isobio (Belgium), 7,10-^{14}C-benzo[a]pyrene (1850 $MBq \cdot mmol^{-1}$, 99.9% of purity) from Amersham (Buckinghamshire, England) and 9-^{14}C-phenanthrene (2035 $MBq \cdot mmol^{-1}$, 98.9% of purity) from Moravek Biochemicals (Brea, USA).

Every contaminated culture medium contained 1258 $Bq \cdot mL^{-1}$ of culture medium (24×10^{-2} $\mu g \cdot mL^{-1}$ for 2,3,7,8-TCDD, 17×10^{-2} $\mu g \cdot mL^{-1}$ for benzo[a]pyrene, 11×10^{-2} $\mu g \cdot mL^{-1}$ for phenanthrene). The compounds were dissolved in DMSO and methanol (Sigma Aldrich, Saint Quentin, France). Methanol and DMSO final concentrations in contaminated and control culture media were respectively 5×10^{-4} and 0.1%, since the work by Carriere et al. [22] ensured that this last concentration had no effect on any parameter of such a study (cell viability, epithelium integrity).

2.2.3. Incubation of the cells with PAHs and 2,3,7,8-TCDD and transfer analysis

All the experiments were conducted at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. After 17 days of culture, as the cells proved to be differentiated, the apical side received a contaminated or a control medium. Exposures were continued for 15, 90, 180 and 360 min. Four repetitions were achieved for each point. At each time and for each molecule, 500 μL of the basal medium of the four culture wells were sampled in triplicate, added with 10 mL of scintillation liquid Ultimagold (Packard, Rungis, France) and counted for 10 min using a Tricarb 460 CD liquid scintillation counter (Packard, Rungis, France). The cells were rinsed with water, scrolled and collected in a solution of soluene (Packard, Rungis, France) and water (8/2). The mixture was kept at 50 °C for 2 h and was eventually counted in liquid scintillation with Hionic Fluor (Packard, Rungis, France). The results were expressed in percentages of the radioactivity dose brought in the apical medium.

2.3. The in vivo model: growing pigs with portal and arterial catheters

2.3.1. Animal surgery

The animal protocol was in accordance with the general guidelines of the Council European Communities [23]. Six castrated Large White pigs (body weight 40 kg) from the herd of a commercial farm were used. Each animal was fitted with one catheter placed in the portal vein and another one in the brachiocephalic artery. Anesthesia was induced with sodium thiopentone (10 to 15 $mg \cdot kg^{-1}$) and maintained with fluothane inhalation (0.5 to 1.5% as required). The animals were fitted with a cuffed endotracheal tube; and the lungs were mechanically ventilated at a minute volume of 150 $mL \cdot kg^{-1}$. Surgery was performed under strictly aseptic conditions. To prevent obstruction by blood clots the cannulaes were rinsed daily with a heparinized (100 $IU \cdot mL^{-1}$) NaCl solution (9 $g \cdot L^{-1}$) under aseptic conditions. The animals began to eat the day after the operation and rapidly re covered their normal growth rate (400 $g \cdot d^{-1}$). They were fed twice a day with a well balanced diet (800 $g \cdot meal^{-1}$) as described by Laurent et al. [24]. Throughout the experimental period, they were kept in individual cages allowing easy access to the cannulae for blood sampling in the brachiocephalic artery and the portal vein.

2.3.2. Preparation of PAHs and 2,3,7,8-TCDD containing milk

The compounds were dissolved in toluene (1.2 mL for 2,3,7,8-TCDD and 1 mL for benzo[a]pyrene) or methanol (1 mL for phenanthrene). The first 10 mL of standardized milk (fat = 4%) were mixed with a labelled molecule and then the radioactive sample was mixed with 990 mL of the remaining milk. Milk contaminated with 2,3,7,8-TCDD and benzo[a]pyrene contained eventually 1850 $kBq \cdot L^{-1}$ (respectively 355 and 235 μg). Contamination with phenanthrene amounted to 555 $kBq \cdot L^{-1}$ of milk (49 μg).

2.3.3. Experimental design

Seven days after surgery, 1 L of milk contaminated with 2,3,7,8-TCDD, benzo[a]pyrene or phenanthrene was given once to two different animals. Ten milliliters of portal and arterial blood were simultaneously collected prior to milk distribution and 1, 2, 3, 4, 5, 6, 9 and 24 h after milk ingestion. The blood samples were immediately centrifuged for 10 min (3000 *g*, 4 °C) and plasma supernatants were stored at –20 °C. At each time and for each molecule, 1 mL of plasma, added with 10 mL of Ultimagold, was counted 10 min with the liquid scintillation counter. Radioactivity was expressed in Bq per mL of plasma. Postprandial kinetics of ^{14}C in the portal vein and arterial blood were determined as well as the postprandial kinetics of the porto-arterial concentration differences. Portal absorption of ^{14}C was calculated as: "^{14}C porto-arterial differences × blood flow". The ^{14}C meal absorption rate was calculated as: "the portal absorption of $^{14}C/^{14}C$ content in the meal". In this study, we calculated portal absorption with a constant blood flow value of 41.2 mL/min/kg body weight, according to Laurent et al. [24].

2.4. Intestinal absorption measurement

The absorption was defined as the percentage of the radioactivity originating from PAHs or 2,3,7,8-TCDD initially brought and measured in the basal compartment for the in vitro model and in the portal blood for the in vivo model.

2.5. Statistical analysis

Percentages of radioactivity counted in the basal medium and in Caco2 cells were analysed with the STATBOX software (Gremmer software) by variance analysis

with total randomization. The model included 2 factors: one time factor (4 independent modalities: 15, 90, 180 and 360 min, each time measure was realized with different wells), one factor molecule (3 independent modalities: 2,3,7,8-TCDD, benzo[a] pyrene, phenanthrene) and the interaction time × molecule. Four repetitions were realized for each point, which meant 4 times × 3 molecules × 4 repetitions = 48 experimental units. Concerning the in vivo data, the results are expressed as the mean of measurements for both animals per molecule.

3. RESULTS

3.1. Intestinal barrier passage

In order to test the effective availability of the molecules for cells, adsorption of the compounds to insert walls was evaluated using hexane. About 1 to 2% of the total radioactivity addition were recovered from the apical walls cleaning after 6 h. This result attested that the molecules were effectively dissolved in the culture medium and available for cells. Furthermore, the basal walls washing provided less than 0.1% of apical original radioactivity. These minor amounts would scarcely influence the kinetics.

Intestinal cell-associated radioactivities allowed to evaluate the percentage of each molecule adsorbed in the cell membranes or in the cell cytoplasm (Fig. 1). The epithelial absorption of the compounds was evaluated thanks to the basal radioactivity measurement (Fig. 2). First of all, the studied micropollutants were able to cross the intestinal barrier. Radioactivity was readily observed in the basal medium after a 15 min incubation.

The cell association profiles and basal appearance rates appeared different according to the studied molecule. For 2,3,7,8-TCDD a "steady state" (plateau) was observed as soon as 90 min. At this time the radioactivity level was about 13% of the initial dose. All along the kinetics, only minor amounts of 2,3,7,8-TCDD were transported in the basal medium, with the values not exceeding 1.5%. The quantity of benzo[a]pyrene bound to cells increased linearly to reach a peak of 39.9% at 180 min. Then, cell radioactivity decreased slowly until the last point. The release of radioactivity originating from benzo[a]pyrene in the basal medium seemed initially very low, but the detected radioactivity significantly

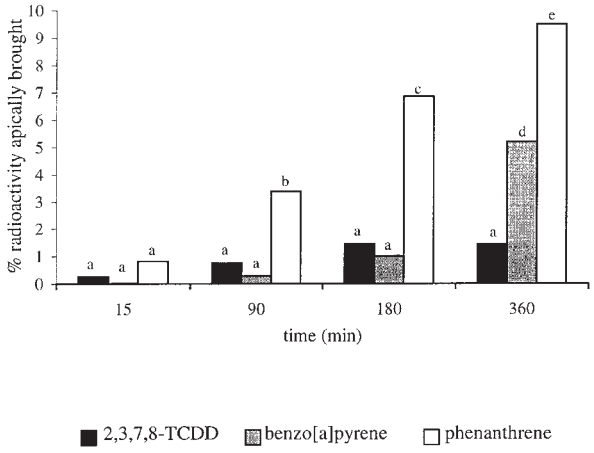

Figure 1. Part of ^{14}C-radioactivity apically brought measured in the basal medium between 15 and 360 min. The columns with the same letter do not differ ($P > 0.05$).

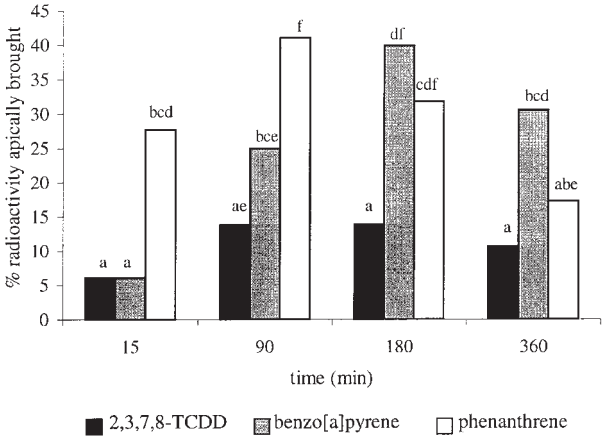

Figure 2. Part of ^{14}C-radioactivity apically brought measured bound to cells between 15 and 360 min. The columns with the same letter do not differ ($P > 0.05$).

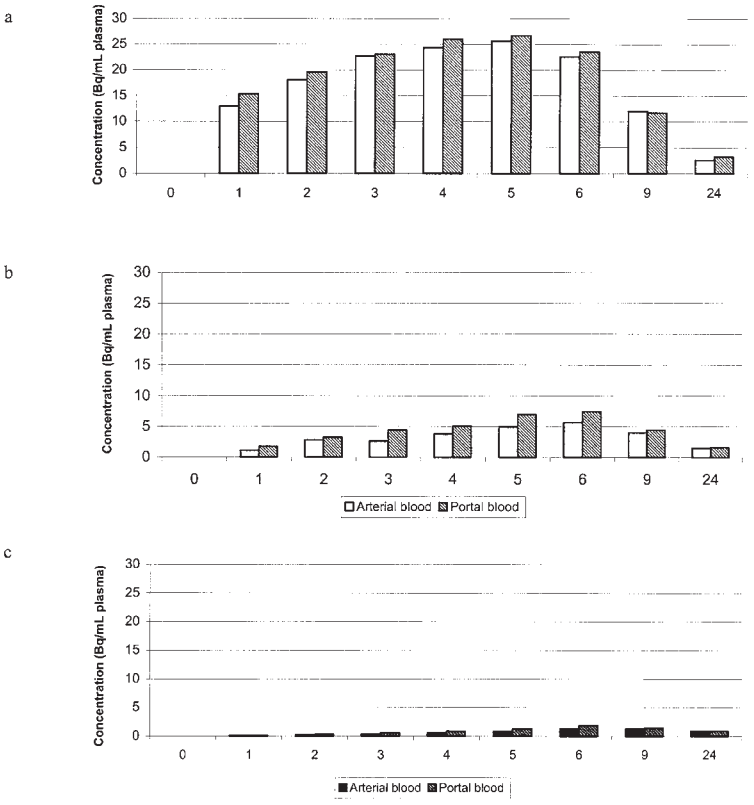

Figure 3. Portal and arterial kinetics of ^{14}C phenanthrene (a), ^{14}C benzo[a]pyrene (b) and ^{14}C 2,3,7,8-TCDD (c) in the growing pig after milk ingestion.

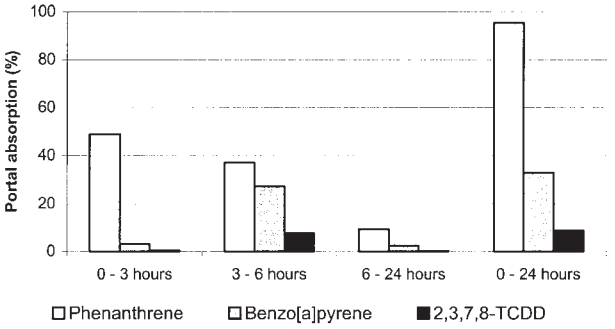

Figure 4. Portal absorption rate of ^{14}C after ingestion by the growing pig of 1000 mL milk spiked with ^{14}C phenanthrene, ^{14}C benzo[a]pyrene or ^{14}C 2,3,7,8-TCDD (mean value, $n = 2$).

increased after 180 min to reach 5% at 360 min ($P < 0.001$). Phenanthrene was more rapidly removed from the culture medium as 28% of its radioactivity dose added apically were found to be associated to cells at 15 min ($P < 0.001$) and it kept on increasing until 90 min to reach 41.1% and then decreased linearly until 360 min where its value was 17.3%. This molecule was significantly more and earlier discharged into the basal medium ($P < 0.001$). The ^{14}C from phenanthrene was finally transported into the basal side about 2 and 7 folds more than from respectively benzo[a]pyrene and 2,3,7,8-TCDD after a 6 h exposure ($P < 0.001$).

3.2. Portal absorption

The pigs were fed with milk containing 2,3,7,8-TCDD, benzo[a]pyrene or phenanthrene. Blood plasma radioactivity was measured to study the porto-arterial kinetics of organic micropollutant absorption (Fig. 3). The label of each studied molecule was transferred from the milk to the blood. The radioactivity originating from the compounds increased rapidly between 1 and 6 h after milk ingestion and then decreased. For each compound, the level of radioactivity in the portal blood was higher than for the brachiocephalic artery (Fig. 4). For phenanthrene associated radioactivity, portal absorption was maximal for the 0–3 h period after milk ingestion whereas for ^{14}C from ^{14}C-benzo[a]pyrene or from ^{14}C-2,3,7,8-TCDD, it was the highest for the 3–6 h period after the milk consumption. Moreover, between 0 and 24 h after milk ingestion, the ^{14}C absorption rate from phenanthrene was respectively about 3 and 10 times more elevated than the levels from benzo[a]pyrene and 2,3,7,8-TCDD (Fig. 4). It should be noticed that the milk radioactivity level from phenanthrene was about 3 times lower than that of benzo[a]pyrene or that of 2,3,7,8-TCDD.

4. DISCUSSION

The present investigation is the first one to study intestinal absorption of PAH and 2,3,7,8-TCDD by both in vitro and in vivo models. Whatever the animal model and the matrix used, they both showed a rapid and a similar differential absorption of the 3 studied organic micropollutants. Absorption of phenanthrene was 2 (with the in vitro model) or 3 folds (with the in vivo model) more elevated than the transfer for benzo[a] pyrene and 7 (with the in vitro model) or 10 (with the in vivo model) folds more than 2,3,7,8-TCDD (Tab. II).

Differential intestinal absorption appeared to be similar considering the in vitro results with the isolated intestinal epithelium and the in vivo results where many microbiological and biochemical interactions take place in the lumen. These observations tend to suggest that the epithelial cells are

Table II. Comparison of the radioactivity absorption rate of ^{14}C phenanthrene, ^{14}C benzo[a]pyrene and ^{14}C 2,3,7,8-TCDD between the in vitro and the in vivo models.

	Phenanthrene	Benzo[a]pyrene	2,3,7,8-TCDD
In vitro	9.5%	5.2%	1.4%
In vivo	86.1%	30.5%	8.3%

one of the main barriers determining the intestinal absorption of the studied organic micropollutants. Even if the unstirred water layer adjacent to the microvillus membrane of the cells created a resistance to absorption increasing with the molecule's lipophilicity [18], the enterocyte culture proved that the studied xenobiotics were able to cross the intestinal barrier without additive molecules like fatty or biliary acids.

Furthermore, at the levels of the intestinal barrier and portal absorption, phenanthrene, the less lipophilic (log Kow = 4.5) and the lightest pollutant, presented the highest absorption, whereas the other compounds saw their passage decreasing in rapidity and quantity as their lipophilicity and their molecular weight increased. Both in vitro and in vivo approaches agreed with some mechanisms of PAHs and 2,3,7,8-TCDD absorption, which seemed at least partially related to their lipophilicity and water solubility. Many works support this hypothesis [25–28]. Moreover, Wils et al. [27] demonstrated in vitro that for molecules with high lipophilicity (log Kow > 3.5), intestinal transport decreases as the lipophilicity increases.

It is interesting to notice the particular behavior of ^{14}C from ^{14}C-2,3,7,8-TCDD. In both experiments, the dioxin showed a lower intestinal absorption than PAHs (about 1.4% in vitro and 8.3% in vivo). Many studies using the balance methods suggested a high digestion and absorption rate of the molecule [7–9]. Our complementary approach considering in vitro and portal absorption seemed rather more precise.

It should be notified that the experiments were carried out with ^{14}C-labelled compounds. The use of radioactivity allowed to follow the absorption of the molecule but did not permit to differentiate the parent molecules and eventual metabolites. Some previous studies suggest an intestinal metabolism of lipophilic micropollutants, especially PAHs [11, 25]. The results presented here probably accounted for the absorption of the molecules but also for their metabolism. This hypothesis should be examined in further work aiming at analysing the compounds studied and their metabolites.

5. CONCLUSION

These two models were suitable to the study of different aspects of the intestinal transfer of organic micropollutants. Both in vitro and in vivo models clearly showed a differential intestinal absorption of the micropollutants. Thus, intestinal cells appear to be one of the main factors governing the absorption of PAHs and 2,3,7,8-TCDD, which seem in part related to the lipophilicity and water solubility of the molecule.

Many regulatory factors were not present in the in vitro model. Biliary acids, present in vivo, have been shown to facilitate the transfer of organic micropollutants by micellar transport [14, 18, 29]. The presence of fatty acids enhances the absorption of hydrophobic pollutants, such as PAHs or polychlorinated biphenyls too [17, 30]. These elements will be added to the culture medium in further experiments, in order to study their effect on intestinal absorption.

Herein, we observed the transfer of radioactivity originating from the micropollutants. Our future works will now use

native molecules in order to clarify the possibility of their biodegradation in the intestinal cells and the consequences on the intestinal absorption.

Abbreviations: BDM: Basal Defined Medium, DMSO: dimethylsulfoxide, EGF: Epidermal Growth Factor, FCS: fetal calf serum, PAHs: polycyclic aromatic hydrocarbons, PBS: phosphate-buffered saline, 2,3,7,8-TCDD: 2,3,7,8-tetrachlorodibenzo-*para*-dioxin.

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