NEW-TOOL TO INVESTIGATE TOXICOLOGICAL HAZARD DUE TO ENDOCRINE DISRUPTORS IN MEDITERRANEAN CETACEANS

M.CRISTINA FOSSI, AND LETIZIA MARSILI

Department of Environmental Sciences, Siena University, Via Mattioli 4, 53100 Siena, Italy.

Contact e-mail:fossi@unisi.it

Abstract

Mediterranean cetaceans, particularly odontocetes, accumulate high concentrations of organochlorine contaminants (OCs) and are therefore exposed to high toxicological risk. Some OCs are known to be endocrine disrupting compounds (EDCs). The hypothesis that Mediterranean cetaceans (*Stenella coeruleoalba, Delphinus delphis, Tursiops truncatus* and *Balaenoptera physalus*) are subject to toxicological risk due to organochlorines and emerging contaminants, such as polybrominated diphenyl ethers (PBDEs) with endocrine disrupting capacity, was investigated using non-lethal "diagnostic" and "prognostic" methods. CYP1A1 activity induction (Benzo(a)pyrene monooxygenase) in skin biopsies was used as a "diagnostic" indicator of exposure to organochlorines in odontocetes and mysticetes and in different populations of *Stenella coeruleoalba*. Marked differences in levels of OCs and CYP1A1 activity were found between fin whales and odontocetes. Organochlorine levels and CYP1A1 activity were significantly higher in the *Stenella coeruleoalba* population of the Mediterranean Whale Sanctuary than in those of two other study areas, suggesting that cetaceans are exposed to high risk in this protected area.

Several questions remain still unanswered in ecotoxicological studies of Mediterranean cetaceans. The need for new biomarkers for EDCs and for a "cell model" to explore the different susceptibilities to several classes of ECDs, including emerging contaminants, led us to culture fibroblasts of different cetacean species as a non-lethal new investigation tool ("dolphins in test tubes"). As a new "prognostic" tool we explored interspecies and gender susceptibility to OC-EDCs and PBDEs using qualitative and semi-quantitative evaluation of target proteins, such as CYP1A1 and CYP 2B in cultured cetacean (*Stenella coeruleoalba, Tursiops truncatus* and *Balaenoptera physalus*) fibroblasts, by western blot, immunofluorescence technique and PCR real time. The information obtained in this pilot experiment will be the basis for further applications and validation of these methodologies to expolore different species and gender susceptibility of marine mammals to different mixtures of endocrine disrupting xenobiotics including emerging contaminants.

KEYWORDS: MEDITERRANEAN CETACEANS, ORGANOCHLORINE CONTAMINANTS; BROMINATED FLAME RETARDANTS; ENDOCRINE DISRUPTING CHEMICALS; FIBROBLASTS CELL CULTURE; IMMUNOFLUORESCENCE TECHNIQUE.

INTRODUCTION

Mediterranean top predators, and particularly cetacean odontocetes, accumulate high concentrations of organochlorine contaminants (OCs) and are therefore exposed to high toxicological risk**.** Some organochlorine compounds, now with worldwide distribution, are known as endocrine disrupting chemicals (EDCs). Four types of organochlorine endocrine disruptors are commonly found in Mediterranean cetaceans (Fossi et al., 2003): 1) environmental estrogens, 2) environmental androgens, 3) anti-estrogens and 4) anti-androgens. The relative estrogenic power of these chemicals, identified by *in vitro* and *in vivo* screening methods is rather weak (10^{-3} or less) compared with the reference power of 17-B-estradiol or DES. However, the high levels of organochlorine compounds detected in Mediterranean cetaceans, and consequently, the high levels of organochlorines with ED capacity, cannot be ignored.

Polybrominated diphenyl ethers (PBDEs) are a major family of brominated flame retardants, and are lipophilic, persistent and toxic to fauna and humans (Alaee et al., 2003). There is growing concern about accumulation of brominated organic compounds in the food chain. The highest levels of PBDEs have been found in top marine predators.

Some general considerations on the potential hazard to these Mediterranean species can be drawn from comparison of the levels of OC-EDCs commonly detected in Mediterranean cetaceans and that of other cetacean species with known reproductive impairment. Several examples suggest that exposure to OC insecticides and PCBs has affected endocrine function and reproduction in marine mammals (Fossi & Marsili, 2003). Alarmingly, levels of PCBs found in the subcutaneous blubber biopsies of Mediterranean free ranging odontocetes sampled in the period 1992-1999 (striped dolphin (*Stenella coeruleoalba*), bottlenose dolphin (*Tursiops truncatus*) and common dolphin (*Delphinus delphis*), mean value = 54587 ng/g l.w.; 44924 ng/g l.w.; 25032 ng/g l.w. respectively) (Fossi et al., 2003) are similar to those detected in the population of beluga whales of the St. Lawrence estuary, including a hermaphrodite specimen (mean value = 78900 ng/g l.w.) (Muir et al., 1996); levels of PCBs

detected in the subcutaneous blubber biopsies of Mediterranean free ranging fin whales (*Balaenoptera physalus*) in the same period (mean value = 7331 ng/g l.w.) (Fossi & Marsili, 2003) are approximately 10 times higher than those found in the population of bowhead whales (*Balaena mysticetus*) characterised by pseudohermaphroditism and other reproductive dysfunctions (mean value = 610 ng/g l.w.) (Hoeskra et al., 2003). These observations suggest the potential risks associated with OC-EDC exposure in Mediterranean cetaceans.

All these considerations orientated our ecotoxicological research in Mediterranean cetaceans towards field application of "diagnostic tools", such as CYP1A1 induction (benzo(a)pyrene monooxygenase activity) in skin biopsies and assay of OC levels in blubber, to assess the exposure of species, populations and genders to OCs with endocrine disrupting capacity. Moreover, the need to develop new biomarkers for EDCs and a cell model ("dolphins in test tubes") to explore different susceptibilities to several classes of EDCs, prompted us to culture fibroblasts of different cetacean species. As "prognostic" tool we explored interspecies (striped dolphin, bottlenose dolphin and fin whale) and gender susceptibilities to OC-EDCs and PBDEs using qualitative and semi-quantitative assay of target proteins, such as CYP1A1 and CYP2B, in cultured cetacean fibroblasts by western blot and immunofluorescence techniques.

MATHERIALS AND METHODS

Sampling. Subcutaneous tissues (skin and blubber) were obtained striped dolphin, bottlenose dolphin and fin whale from the western Ligurian Sea, between Corsica and the French-Italian coast, and the Ionian Sea using an aluminium pole armed with biopsy tips or biopsy darts launched with a crossbow (Barnett Wildcat II crossbow with a 150-pound test bow) (Fossi et al, 2003). Biopsy specimens were taken in the dorsal area near a dorsal fin and on the upper part of the caudal peduncle. All material was immediately placed in liquid nitrogen or stored in cell medium.

Sex identification. Cetacean gender was determined genetically according to Berube & Palsboll (1996).

CYP1A1 activity. The small size of the biopsy samples (0.200 - 0.002 g) did not permit isolation of microsomal fractions. CYP1A1 activity (BPMO) was detected in whole tissue. Since the connective tissue was very tough, the epidermis was homogenized in 1.15% KCl buffer at pH 7.5 by thermal shock and separated by freezing in liquid N_2 and pulverizing in a Potter apparatus

with ultrasound. BPMO activity was assessed using the incubation mixture proposed by Fossi et al. (1992) incubating each sample (plus blanks) in a shaking bath for 2 h at 37°C. The activity was expressed in arbitrary units of fluorescence (A.U.F./h/g tissue).

Organochlorines. The samples of subcutaneous blubber (about 0.3 g) were freeze-dried and extracted with n-hexane in a Soxhlet apparatus for analysis of chlorinated hydrocarbons. The analytical method was High Resolution Capillary Gas Chromatography with a Perkin-Elmer Series 8700 GC and a 63Ni ECD. Capillary gas-chromatography revealed op'- and pp'- isomers of DDT and its derivatives DDD and DDE, and about 30 PCB congeners.

Fibroblasts cell culture. The development of a non-invasive sampling method for obtaining viable tissue samples for cell cultures from skin biopsies of free-ranging cetaceans was described in Marsili et al. (2000). Successful cell cultures were obtained from striped dolphin, bottlenose dolphin and fin whale. The first fibroblasts were observed after 7–21 days. Cultures reached 90% confluence in 15–20 days, when they were trypsinized, washed and placed in Falcon 50 and 125 flasks, after two and three trypsinizations respectively.

Experimental design. Fibroblast cultures (third generation) from striped dolphin (n=15), bottlenose dolphin (n=2) and fin whale (n=3) were subject to two different experimental protocols for 48 h, using two classes of CYP450 inducers with EDCs potency. The first was a mixture of Arochlor 1260, pp'DDT and pp'DDE in DMSO (0.05%) at three doses: 1 µg/ml, 5 µg/ml and 25 µg/ml, plus a DMSO (0.05%) control. The second was a mixture (BDE-MXE, Wellington, Canada) containing 27 PBDEs, from mono- to deca-brominated, in nonane (0.01 µg/ml) at three doses: 0.1 µg/ml, 0.05 µg/ml and 0.01 µg/ml, plus a nonane (0.01 µg/ml) control.

Western blot. For western blot analysis, fibroblast extracts were separated by SDS-PAGE (10% polyacrylamide gels) and blotted into nitrocellulose sheets for 1 hour at a constant voltage of 100 V. The membranes were saturated by incubating with blocking solution (2% BSA in TTBS) for 1 hour at room temperature. Primary polyclonal goat IgG anti rabbit antibodies CYP1A1/1A2 and CYP2B4 were purchased from Oxford Biomedical Research (Michigan, USA). CYP1A1/1A2 and CYP 2B4 diluted 1:5000 and 1:1000 respectively in TTBS-1% BSA, were allowed to incubate for 15 h at 4°C. Incubation with the BioRad anti-goat HRP labelled secondary antibody (1:3000 final dilution) was performed for 1 hour at room temperature and detection was carried out as outlined in the Amersham ECL kit booklet. Semi-quantitative analysis was performed with Quantity One software (Bio-Rad).

Immunofluorescence. We used immunofluorescence in fibroblast cultures for a qualitative and semi-quantitative analysis of target proteins CYP1A1 and CYP2B. After a first reaction with the primary antibodies for CYP1A1-1A2 and 2B4 (Oxford Biomedical Research)**,** the cells were treated with the respective secondary antibodies marked with a fluorochrome.

RESULT AND DISCUSSION

In the last 15 years, in our Lab, we have mainly based ecotoxicological research in Mediterranean cetaceans on "diagnostic tools", namely CYP1A1 induction (benzo(a)pyrene monooxygenase activity) in skin biopsies and quantification of OCs in blubber, to assess different exposure of species, populations and genders to OCs in the Mediterranean Sea (Fossi et al., 1992; Marsili et al., 1998; Fossi et al., 2003; Fossi & Marsili 2003). Several questions were solved, other are still open.

With regard to species differences, we evaluated CYP1A1 (BPMO) activity in skin biopsies of Mediterranean cetaceans (striped dolphin, bottlenose dolphin, common dolphin and fin whale) as a potential indicator of exposure to EDCs, such as OCs. In line with data in the literature and results obtained in our lab before 1994 (Fossi et al., 1992; Marsili et al. 1998; Fossi et al., 2003; Fossi & Marsili 2003), sharp differences in subcutaneous blubber levels of all contaminants were found between fin whales (PCBs mean value=4.5 ng/g f.w; DDTs mean value=5.3 f.w) and odontocete species (in striped dolphin, PCBs mean value=19.5 ng/g f.w; DDTs mean value=20.1 f.w). The same was found for BPMO activity (fin whale, mean value=140.5 U.A.F. g tissues/h; striped dolphin, mean value=270.2 U.A.F. g tissues/h). The main explanation for these results is their different position in the food chain: odontocetes are terminal consumers and fin whales are macroplanktophages.

Sex differences in CYP1A1 (BPMO) induction were also investigated. In striped dolphins a linear correlation was found between op'DDT/BPMO and PCB153/BPMO (Fossi et al., 2003). In the common dolphin five linear correlations with the BPMO activity were identified: DDTs, pp'DDE, op'DDT, PCBs and PCB153. The main result in this species was absence of induction of BPMO in females with increasing levels of contaminants. A similar result was obtained in fin whales sampled in the Ligurian Sea from 1992 to 1995 (Marsili et al., 1998). This difference in the inductive capacity of skin CYP1A1 (BPMO) between males and females of this species was interesting but at that stage could not be explained.

With regard to population differences, organochlorine levels and CYP1A1 activity were significantly greater in the striped dolphin population of the Mediterranean Whale Sanctuary

than in those of two other Mediterranean study areas, suggesting that cetaceans are exposed to high risk in this protected area.

Several questions remain unanswered in ecotoxicological studies of Mediterranean cetaceans. The need for new biomarkers for EDCs and a "cell model" to explore the different susceptibilities to several classes of ECDs led us to culture fibroblasts of different cetacean species ("dolphins in test tubes"). Here we propose and apply three new methodological tools to detect cultured fibroblast responses to OC-EDCs and PBDEs: immunofluorescence technique, western blot and real time PCR. We discuss the preliminary results of the first two. As "prognostic" tool we explored interspecies and gender susceptibility to OC-EDCs and PBDEs using qualitative and semi-quantitative evaluation (western blot and immunofluorescence) of the target proteins CYP1A1 and CYP2B in cultured fibroblasts.

Particular attention was paid to the role of detoxification enzymes (CYP2B) and the related biochemical susceptibility of the different species to different classes of chemicals. The role of CYP2B *in vitro* metabolism of two tetrachlorobiphenyl congeners were previously studied in beluga and pilot whale (White et al., 2000).

The main results of these pilot experiments using this non-lethal new investigation tool were the followings.

1) The detection of CYP 1A1-1A2 (Fossi personal comunication) and CYP 2B4 in bottlenose dolphin, striped dolphin and fin whale fibroblasts, revealed by fibroblast immunofluorescence (Figure 1 A, B, C) and by western blot analysis (Figure 2).

2) Different increases in fluorescence (2B cytochromes) was found between odontocetes and mysticetes in relation to contaminant doses, with higher induction responses in striped dolphin and particularly bottlenose dolphin (Figure 1 A, B) than in fin whale (Figure 1 C);

3) A gender-related different patterns of induction (cytochromes 2B) of striped dolphin (Figure 2A) were also found, with higher response capability in male than in female.

4) Increasing doses of contaminants produced increasing induction of CYP2B4, as revealed by both methodologies (Figure 1,2). Greater induction by PBDE than by OC treatment in bottlenose dolphin were detected (Figure 2 B). In particular the highest treatment dose of

PBDE, 250 time lower than OCs, was able to produce an induction phenomenon about two times higher than OCs. These data represent a first worning of the *in vitro* high toxicological potential of this emerging chemicals (PBDE) in cetaceans.

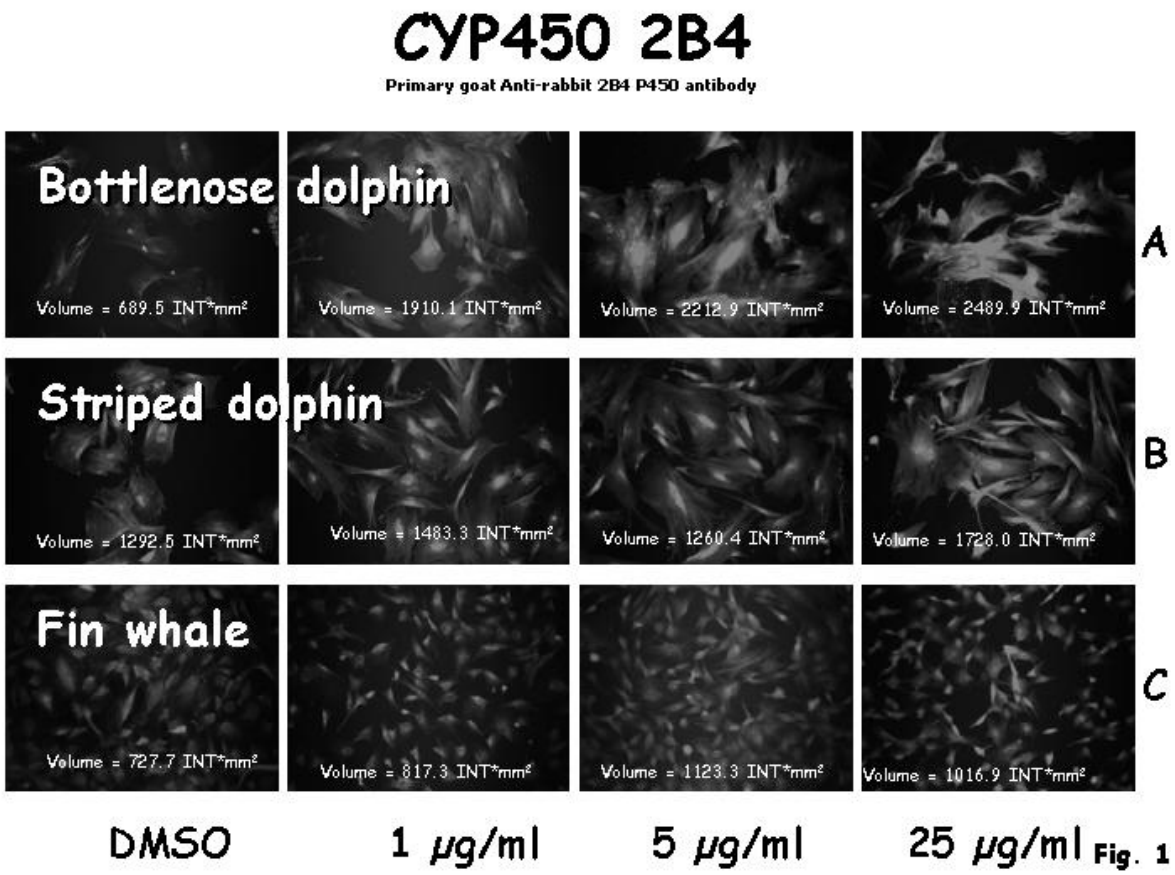

Figure 1- Immunofluorescence: cultured fibroblast from bottlenose dolphin (**A**), striped dolphin (**B**), and fin whale **(C),** treated for 48h with a mixture (1, 5, 25 µg/ml) of Arochlor 1260, pp'DDT and pp'DDE in DMSO. CYP2B4 primary goat anti-rabbit 2B4 P450 antibody was from Oxford Biomedical Research. Arithmetic mean of Relative Volume Intensity (INT*mm2) are reported for each slides.

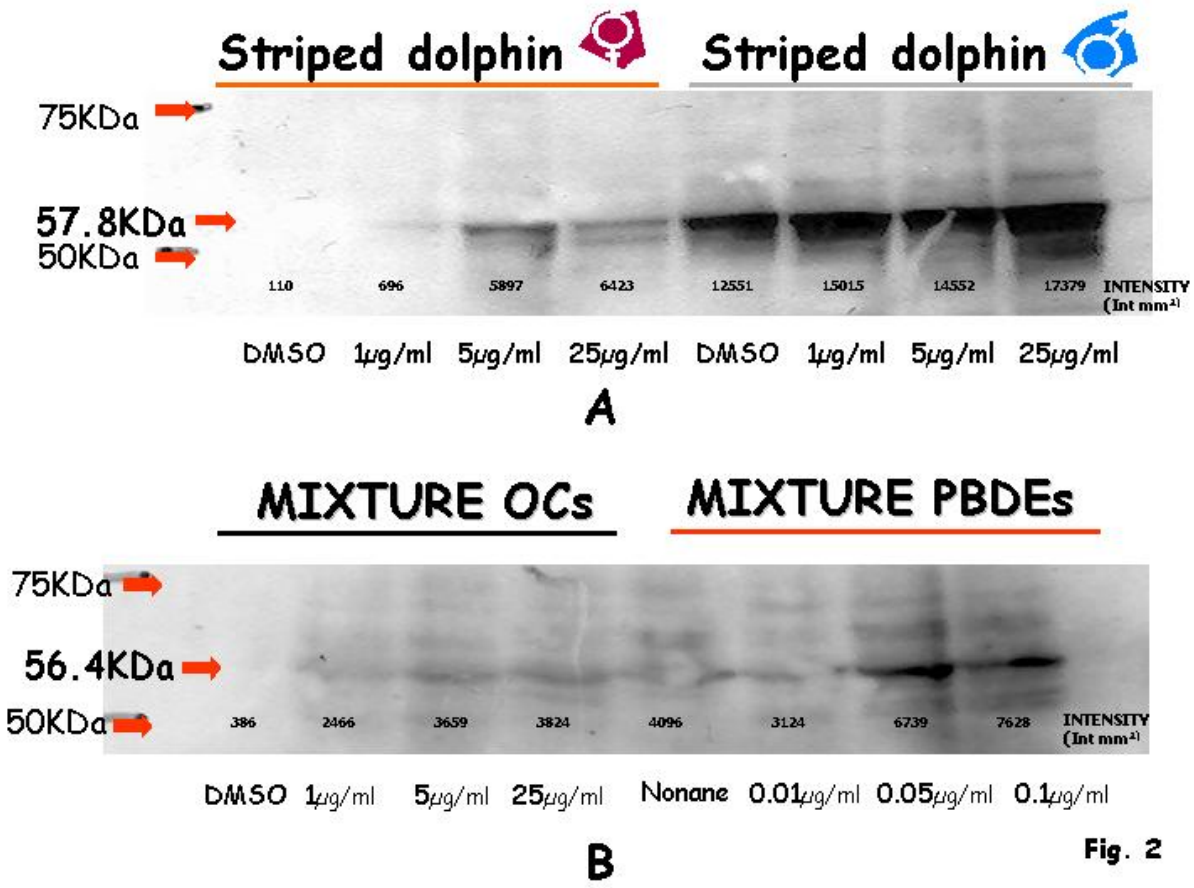

Figure 2. Western blot analysis of CYP2B4 in fibroblast cell culture of **(A)** striped dolphins (male and female) treated for 48h with a mixture (1, 5, 25 µg/ml) of Arochlor 1260, pp'DDT and pp'DDE in DMSO. Western blot analysis of CYP2B4 in fibroblast cell culture of (**B**) bottlenose dolphin treated for 48h with two mixture: a mixture (1, 5, 25 µg/ml) of Arochlor 1260, pp'DDT and pp'DDE in DMSO; a mixture of 27 PBDEs (0.01, 0.05,0.1 µg/ml), from mono- to deca-brominated, in nonane. CYP2B4 primary goat anti-rabbit 2B4 P450 antibody was from Oxford Biomedical Research. Arithmetic mean of Relative Volume Intensity (INT*mm2) are reported for each lanes.

In conclusion the information obtained in this pilot experiment will be the basis for further applications and validation of these methodologies (immunofluorescence, western blot in cultured cetaceans fibroblasts), integrated with gene expression studies (by real time PCR), to expolore different species and gender susceptibility of marine mammals to different mixtures of endocrine disrupting xenobiotics including emerging contaminants.

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