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Short communication

Development of new-tools to investigate toxicological hazard due to endocrine disruptor organochlorines and emerging contaminants in Mediterranean cetaceans

M. Cristina Fossi *, Letizia Marsili, Silvia Casini, Daniela Bucalossi

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

Abstract

The possibility that certain Mediterranean cetaceans are subject to toxicological hazard due to organochlorines and emerging contaminants, such as polybrominated diphenyl ethers (PBDEs) with endocrine disrupting capacity, was investigated using non-lethal methods. 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 ("dolphins in test tubes"). We then explored interspecies and gender susceptibility to OC-EDCs and PBDEs using qualitative and semi-quantitative evaluation of target proteins, such as CYP1A and CYP2B in cultured cetacean fibroblasts (*Stenella coeruleoalba*, *Tursiops truncatus* and *Balaenoptera physalus*), by western blot and immunofluorescence techniques.
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Keywords: Mediterranean cetaceans; Organochlorine contaminants; Brominated flame retardants; Endocrine disrupting chemicals; Fibroblasts cell culture; Immunofluorescence technique; Western blot

Mediterranean top predators, and particularly cetacean odontocetes, accumulate high concentrations of organochlorine contaminants (OCs) and this exposure may increase their risk of disease. Some organochlorine compounds are also known as endocrine

* Corresponding author.
E-mail address: Fossi@unisi.it (M.C. Fossi).

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disrupting chemicals (EDCs) (Fossi et al., 2003). 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. In fact several examples suggest that exposure to levels of OC insecticides and PCBs, commonly detected in Mediterranean odontocetes, has affected endocrine function and reproduction in other marine mammal species (Fossi and Marsili, 2003).

Moreover there is growing concern about accumulation and effects of emerging contaminants such as polybrominated diphenyl ethers (PBDEs) (a major family of flame retardants) in the food chain. PBDEe are lipophilic, persistent and toxic to fauna and humans (Alaee et al., 2003). The highest levels of PBDEs have been found in top marine predators, including Mediterranean odontocetes.

All these considerations orientated a decade of our ecotoxicological research in Mediterranean cetaceans towards field application of a powerful indicator of exposure to lipophilic contaminants, 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, 2003; Marsili et al., 1998; Fossi and Marsili, 2003). 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"). In this study we evaluated three methodologies to detect cultured fibroblast responses to OC-EDCs and PBDEs: immunofluorescence technique,western blot and real time PCR. Here we present the preliminary results of the first two. We explored interspecies and gender susceptibility to OC-EDCs and PBDEs using qualitative and semi-quantitative evaluation of the target protein CYP2B in cultured cetaceans fibroblasts (*Stenella coeruleoalba*, *Tursiops truncatus* and *Balaenoptera physalus*). 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 *in vitro* metabolism of two tetrachlorobiphenyl congeners were previously studied in beluga and pilot whale (White et al., 2000).

Sampling: Skin and blubber samples were obtained in striped dolphin, bottlenose dolphin and fin whale from the western Ligurian Sea, between Corsica and the French-Italian coast, using an aluminium pole armed with biopsy tips or biopsy darts launched with a crossbow (Fossi et al., 2003). All material was immediately placed in liquid nitrogen or stored in cell medium.

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

Fibroblasts cell culture: The development of a non-lethal 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 subjected to two different

experimental protocols for 48 h, using two classes of CYP inducers with EDCs potency. The first was a mixture of OCs (Arochlor 1260, *pp′*DDT and *pp′*DDE at 8.5 μg/ml each 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 (IUPAC Isomers 3, 7, 15, 17, 28, 47, 49, 66, 71, 77, 85, 99, 100, 119, 126 at 1 μg/ml, 138, 153, 154, 156, 183, 184, 191, 196, 197 at 2 μg/ml and 206, 207, 209 at 5 μg/ml) 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 onto nitrocellulose sheets for 1 h at a constant voltage of 100 V. The membranes were saturated by incubating with blocking solution (2% BSA in TTBS) for 1 h at room temperature. Primary polyclonal goat IgG anti-rabbit P450 2B4 antibody was purchased from Oxford Biomedical Research (Michigan, USA). P450 2B4 diluted 1:1000 in TTBS-1% BSA, was allowed to incubate for 15 h at 4 °C. Incubation with the Bio-Rad anti-goat HRP labelled secondary antibody (1:3000 final dilution) was performed for 1 h 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). Results were expressed as relative volume intensity mm^2 ($INT * mm^2$). Data were analyzed using non-parametric statistic of Kolmogorov–Smirnov.

Immunofluorescence: We applied immunofluorescence in fibroblast cultures for qualitative and semi-quantitative analysis of the target protein CYP2B. After a first reaction with

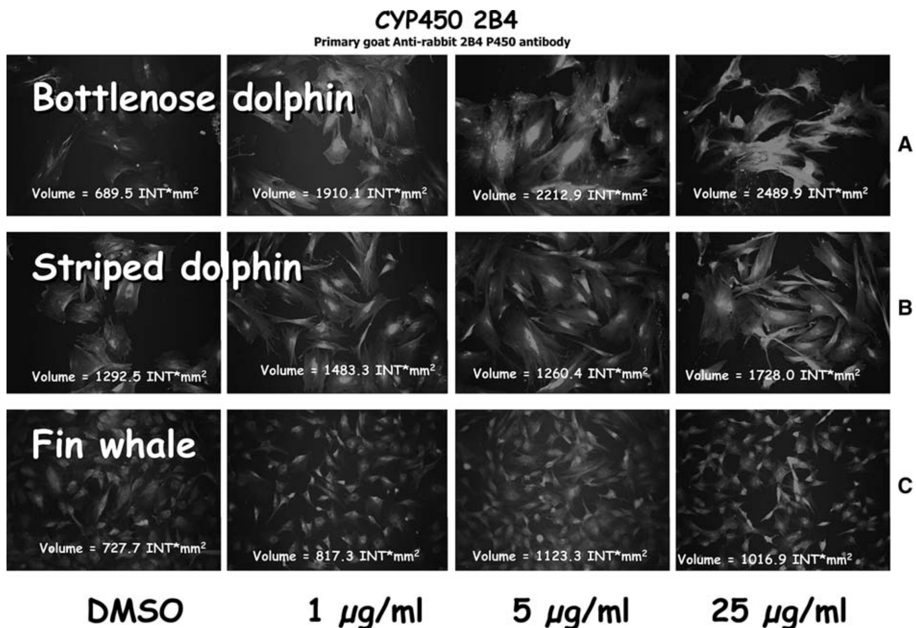

Fig. 1. Immunofluorescence: cultured fibroblast from bottlenose dolphin (A), striped dolphin (B), and fin whale (C), treated for 48 h with a mixture (1, 5, 25 μg/ml) of Arochlor 1260, *pp′*DDT and *pp′*DDE in DMSO. CYP2B4 primary goat anti-rabbit 2B4 P450 antibody was from Oxford Biomedical Research.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

the primary antibodies for CYP 2B4 (Oxford Biomedical Research), the cells were treated with the respective secondary antibodies marked with a fluorochrome.

Semi-quantitative analysis was performed (five slides for each treated sample) with Quantity One software (Bio-Rad). Results were expressed as relative volume intensity mm^2 ($INT * mm^2$). Data were analyzed using non-parametric statistic of Kolmogorov–Smirnov.

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

(1) The detection of CYP2B in bottlenose dolphin, striped dolphin and fin whale fibroblasts, revealed by fibroblast immunofluorescence (Fig. 1A–C) and by western blot analysis (Fig. 2).
(2) Different increases in fluorescence (CYP2B) was found between odontocetes and mysticetes in relation to contaminant doses, with higher induction responses in striped dolphin and particularly bottlenose dolphin (Fig. 1A, B) than in fin whale (Fig. 1C).
(3) Gender-related patterns of induction (CYP2B) of striped dolphin (Fig. 2A) were also found, with higher response capability in males than in females.
(4) Increasing doses of contaminants produced increasing induction of CYP2B, as revealed by both methodologies (Figs. 1 and 2). Greater induction by PBDE than by OC treatment in bottlenose dolphin were detected (Fig. 2B). 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 warning of the *in vitro* high toxicological potential of this emerging chemicals (PBDE) in cetaceans.

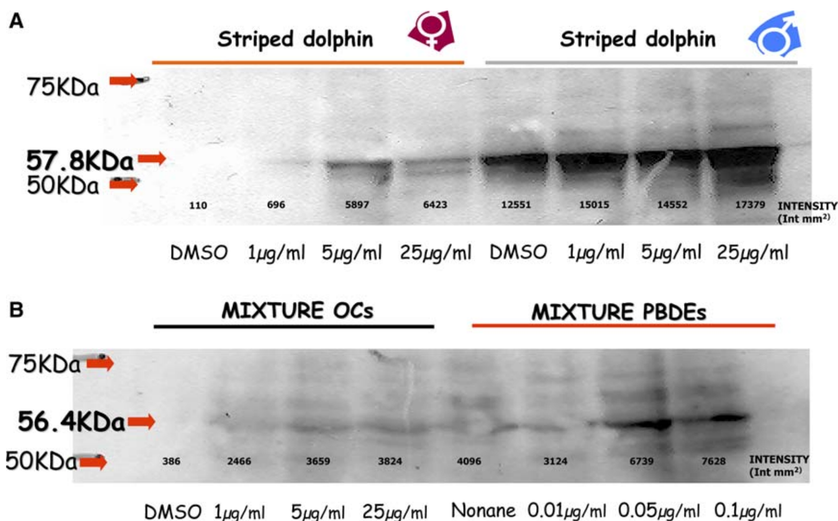

Fig. 2. Western blot analysis of CYP2B in fibroblast cell culture of (A) striped dolphins (male and female) treated for 48 h with a mixture (1, 5, 25 μg/ml) of Arochlor 1260, *pp′*DDT and *pp′*DDE in DMSO. Western blot analysis of CYP2B4 in fibroblast cell culture of (B) bottlenose dolphin treated for 48 h 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 ($INT * mm^2$) 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 explore different species and gender susceptibility of marine mammals to different mixtures of endocrine disrupting xenobiotics including emerging contaminants.

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References

Alaee, M., Arias, P., Sjödin, A., Bergman, A., 2003. Environ. Int. 29, 683–689.

Berube, M., Palsboll, P., 1996. Mol. Ecol. 5 (2), 283–287.

Fossi, M.C., Marsili, L., 2003. Endocrine disruptors in aquatic mammals. SCOPE/IUPAC. Pure Appl. Chem. 75, 11–12. 2 235–2247.

Fossi, M.C., Marsili, L., Leonzio, C., Notabartolo di Sciara, G., Zanardelli, M., Focardi, S., 1992. Mar. Pollut. Bull. 24 (9), 459–461.

Fossi, M.C., Marsili, L., Neri, G., Natoli, A., Politi, E., Panigada, S., 2003. Mar. Pollut. Bull. 46, 972–982.

Marsili, L., Fossi, M.C., Notarbartolo di Sciara, G., Zanardelli, M., Nani, B., Panigada, S., Focardi, S., 1998. Chemosphere 37 (8), 1501–1510.

Marsili, L., Fossi, M.C., Neri, G., Casini, S., Gardi, C., Palmeri, S., Tarquini, E., Panigada, S., 2000. Mar. Environ. Res. 50 (1–5), 649–652.

White, R.D., Shea, D., Schlezinger, J.J., Hahan, M.E., Stegemann, J.J., 2000. Comp. Biochem. Physiol., Part C 126, 267–284.