Potential use of Vitellogenin and Zona radiata proteins as biomarkers of endocrine disruption in Peregrine falcon exposed to organochlorine compounds (DDTs, PCBs, PCDDs and PCDFs).

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Introduction

Many different classes of environmental contaminants such as industrial chemicals (e.g. alkylphenols, polychlorinated biphenyls, pesticides, PAHs, polychlorinated dibenzo-p-dioxins, and dibenzofurans), "can cause adverse effects in the reproductive functions of intact organisms or their progenies, consequent to changes in endocrine functions" showing a so-called Endocrine disruptor activity[1]. Avian raptor species, such as peregrine falcon (*Falco peregrinus*) for their peculiar position in the food web are potentially at risk in relation to the accumulation of Persistent Organic Pollutants (POPs) and toxic metals[2-4].

Recent studies carried out with Peregrine falcon (*Falco peregrinus*) in Spain reveal a contamination with organochlorine compounds (PCDDs, PCDFs, PCBs and DDTs) which could be responsible of the decrease of successful pairs observed during the last ten years[5]. Thus there is a need to develop sensitive diagnostic monitoring tools for the evaluation of toxicological risk and potential effects on the reproductive function and population dynamic of avian top predator species. Two markers for the detection of EDs effects in oviparous vertebrates are induction of Vitellogenin (Vtg) and Zona Radiata Proteins (ZR). Vtg, a complex phospholipoglycoprotein, is the major egg-yolk protein precursor and is normally synthesized by females in response to estradiol. ZR together with Zona Pellucida (ZP) constitutes in birds part of the eggshell. These proteins (Vtg, ZR and ZP) are normally synthesised in the liver as a response to an estrogen signal given by Estradiol. Males and sexually undifferentiated specimens also have the Vtg and ZR genes but do not express them unless exposed to estrogenic compounds.

The main aim of this preliminary study was to develop methods for the detection of Vtg and ZR in plasma obtained from peregrine falcon as a specific biomarker for the evaluation of the effects of EDCs.

Material and Methods

The induction of VTG and ZR in plasma samples from the 4 sexually mature falcons; two males (M1, M2) and two females (F3, F4) were assayed. Total plasmatic concentration of proteins was measured using the method of Bradford[6].

Four Polyclonal Antibodies against Fish Vtg (Biosense, Norway), AA1, PO2, CS1, CS2 and two against Fish ZR, O146, O173, were screened in ELISA assay and WESTERN BLOT. Indirect ELISA was performed according to the method of Goksoyr[7]. 10 μg of total protein/well were assayed. Primary polyclonal rabbit antibodies anti Vtg (anti salmon AA-1, anti sea bream PO-2, anti cod CS-1 and anti turbot CS-2) and anti ZR (anti salmon O146 and O173) were diluted 1:1000. Secondary antibody "goat anti rabbit Ig G (H+L)" was diluted 1:3000. Results were expressed as absorbance at 490 nm wavelength.

Western blot was performed according to the method of Laemmli[8] and Towbin[9]. Aliquots (20 ug plasma protein) were loaded on 10% (for the determination of ZR) and 7,5% (for Vtg). SDS gels and separated at constant voltage of 140 V for 1 h. Thereafter proteins were transferred to a nitrocellulose membrane (0,45 mm, BioRad) for 1 h at constant voltage of 100 V. The membrane were saturated by incubating with blocking solution (3% gelatine dissolved in Tris buffered saline containing 0.05% tween-20 TTBS). Primary polyclonal rabbit antibodies anti Vtg (anti salmon AA-1, anti sea bream PO-2, anti cod CS-1 and anti turbot CS-2) were diluted respectively 1:1000, 1:1000, 1:500 and 1:500). Antisera anti ZR (O146 and O173) were diluted 1:1000. Secondary antibody "goat anti rabbit Ig G (H+L)" was diluted 1:3000. The developing of the nitrocellulose membrane was carried out using the enhanced chemiluminescence system based on horseradish peroxidase and luminol/oxidant/enhancer reagent (ECL)[10].

Molecular mass was estimated using the following markers: myosin (200 kDa), β galactosidase (175 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), Lysozyme (16,5 kDa), Aprotinin (6,5 kDa) from BioRad.

Results and discussion

The total concentration of proteins in plasma ranged from 40 to 55 mg/mL of plasma. A significant difference between males and females was found. In tables 1 and 2 are reported the results of ELISA assay: different degrees of cross-reactivity were found. The polyclonal rabbit antibodies AA1 anti salmon, CS2 anti turbot and PO2 anti seabram showed a much wider cross-reactivity in female falcons. An apparent induction of Vtg in males was also found. CS1 anti cod Vtg showed a very low cross-reactivity. Both O-146 and O-173 showed a wide cross-reactivity in female falcons while a low induction of ZR was found in males. WESTERN BLOT substantially gave more informations about the nature of the proteins detected with ELISA. Different degrees of specificity for the antibodies tested were found. Anti seabream Vtg PO2 showed a specific reaction binding with high affinity to polypeptides having molecular masses ranging from 70 to 65 kDa in female falcons (F1, F2) and probably referred to lipovitellin and other yolk proteins.

Table 1. ELISA results for Vtg screening in plasma of peregrine falcon (Abs 490 nm).

Anti Vtg Antibodies	Salmon	Seabream	Cod	Turbot
	AA1	PO2	CS1	CS2
M1	0,26	0,04	0,04	0,11
M2	0,13	0,06	0,04	0,10
F1	0,043	1,05	0,37	1,02
F2	0,15	0,06	0,30	0,054

Table 2. ELISA results for Zrp screening in plasma of peregrine falcon (Abs 490 nm).

Anti ZR Antibodies	Salmon	
	O-173	O-146
M1	0,19	0,11
M2	0,17	0,10
F1	0,17	0,11
F2	0,14	0,02

A similar reaction was lightly observed in males (M1, M2) (figure 1). Both anti ZR antiserum O173 and O146 showed high affinity to a polypeptide having a molecular mass of about 40 KDa and probably referred to ZR or ZP proteins. Anti salmon AA1, anti cod CS1 and anti turbot CS2 did not show a significant cross-reaction (results not shown).

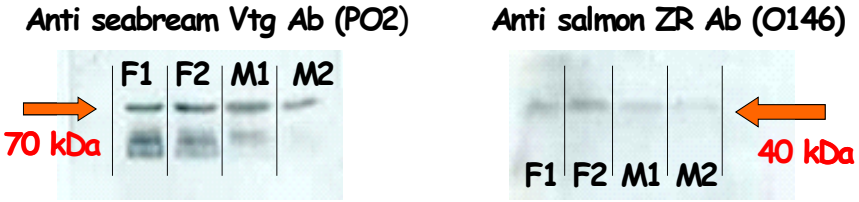

Figure 1. WESTERN BLOT results for Vtg (PO2) and Zrp (O146) screening in plasma of peregrine falcon

In conclusion this study shows that Rabbit Anti seabream Vtg polyclonal antibody (PO2) seems to be the more specific in binding plasmatic lipoproteins in peregrine falcon. Both rabbit anti salmon ZR polyclonal antibodies (O146 and O173) clearly binds to ZR and ZP in peregrine falcon. These preliminary results confirm the applicability of both these diagnostic tools (induction of Vtg and ZR) in detecting exposure to EDCs in this species. Moreover the apparent induction of Vtg and ZR detected in male specimens suggest a potential hazard to EDCs in the peregrine falcon which represents a species still affected by organochlorine compounds, in particular those with estrogenic activity.

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