ANGLISTICUM
International Journal of Literature, Linguistics & Interdisciplinary Studies

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Detection of the Effect of the Action of Pesticides, Herbicides and Fungicides in the Liver Fish *Barbus Peloponnesius* through Biological Markers as Pollutant Indicators of the Water of the Vardar River

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Received: 30 January 2013; **Revised:** 10 February 2013; **Accepted:** 18 February 2013

Abstract: Fish as bio indicators have a very important role in monitoring the pollution of aquatic biotopes, as they are much sensitive to the presence of ksenobionts in the aquatic environments. Water pollution with ksenobionts (industrial pollutants, agricultural pollutants, etc.), affects in the metabolic processes of the aquatic organisms. After the penetration and absorption of ksenobit from the body, they reach biotransformation, or get conjugated with the specific structures of the cell such as specific receptors located in the peripheral part of the cell, the cell membrane and inside its cytoplasm and also in the cell organelles. ksenobiont's connection with membrane receptors can initiate cellular processes that have toxic effects or other adverse effects on the cell itself. Such processes have negative effects in the organism which afterwards these are affected with the body organs or the whole population. As meaningful indicators that can determine the detection of ksenobionts in the aquatic environment of fish are biological markers. As a safe and adequate biomarker to determine the pollution of aquatic biotopes is cytochrome P4501A (Anzenbacherova and Anzenbacher 1999).

Keywords: fish, ksenobiontët, biomarker, cytochrome P4501A, receptor-AH etc.

Introduction

In the last forty years pesticides have become an inevitable part of their use in the world's agriculture. Recently, many efforts have been given to identify new pesticides and with greater efficacy for the treatment of agricultural crops. Aquatic environment turned into a place where repeatedly ksenobionts and pollutants act, such as polychlorinated biphenyls (PCBs) and chlorinated pesticides. An efficient monitoring system which indicates the presence of these ksenobionts in the aquatic environment is cytochrome P450. Cytochrome P450 is very important biochemical marker and as an indicator of some certain pollutants in the aquatic environment (Jung et al., 2001). This biomarker is affected to the wide range of industrial pollutants, e.g. dioxins, polychlorinated biphenyls, polycyclic aromatic hydrocarbures (Van der Oost et al., 2001; White et al., 2003). Such pollutants accumulate in large concentrations in the rivers sediments and from there through the food chain penetrate in the aquatic organisms (Malins et al., 1984). Pesticides and many other ksenobionts act as substrates, inhibitor or inductor of the enzymes that metabolise drugs, including cytochrome P-450s (CYP) (Hodgson and Levi, 1996). With previous researches was reached the acquaintance for the inductive effect of insecticides, different isoenzymes CYP (Campbell et al., 1983; Li et al., 1995) and the acquaintance to regulate the expression of CYP genes in the targeted tissues, such knowledge represents a powerful tool for assessing the health situation and assessing the pollution of the aquatic environment. Induction of CYP1A1 has also been used to monitor the potential acute and chronic toxicity of insecticides (Delescluse et al., 1998; Dubois et al., 1996).

Induction of cytochrome-P450

Induction of cytochrome-P450-AH is mediated by the AH receptor, a protein which binds the present ksenobionts in the cellular cytoplasm (Lewis 2001; Sadar and Andersson 2001; schlenk and Di Giulio 2002; Billiard et al., 2002).

So far the investigations have shown that the first system (cytochrome P4501 A1), toxic chemical material is bound to chemical-AH receptor in the cytoplasm of the cell and releases the HSP 90, which is then replaced with tRNAs (Fig. 1).

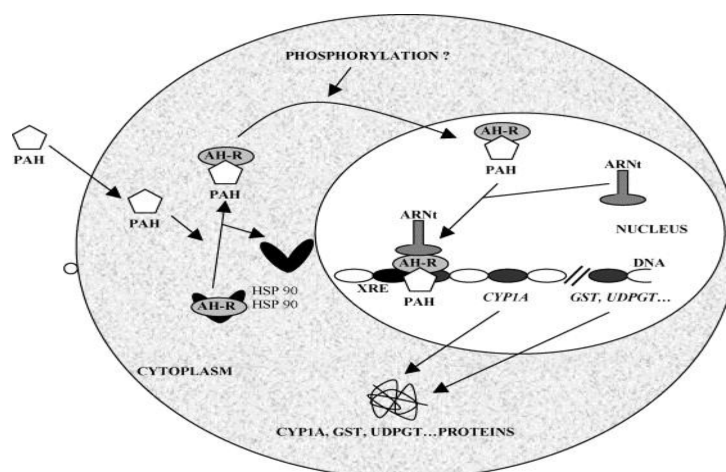

Fig.1.Schematic representation of ksenobiont's action after penetration into the cell

AH-R (Aril hydrocarbons Receptor), HSP90 (Heat Shock Protein 90 kDa); tRNAs (nuclear translocator Ah-receptor), XRE (Ksenobiotic Responsive Elements), PAH (Planar Aromatic Hydrocarbons), CYP1 (Cytochrom of the P4501A), GST (Glutation-S-Transferase), UDPGT (UDP-glucuronil transferase)

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This new compound enters the cell nucleus and binds DNA specifically, enabling it access towards transcription factors, which are responsible for the cytochrome P450 1A1 gene transcription, which through mRNA, passes into cytoplasm and then are transported to the endoplasmic reticulum which leads to the degradation of toxic substances. Pollutants with greater ability to connect to the AH receptor, also have greater capacity to induce CYP 1A, but with adverse consequences (Billiard et al., 2002).

Material and methods

As experimental animals for the realization of this study is to be used the type of fish called Barbus peloponnesius (Valenciennes, 1842) caught in several locations of the Vardar River, through "electroshock" method, using the CYBER POWER generator. This type of fish, hunt in this way, are thrown into a mobile aquarium supplied with air pump, and then are transferred to the Medical Scientific Research Laboratory aquariums. Aquariums in which there were kept the fish are of size 75x35x40, water temperature 16^{0}C and with the oxygen level of 6mg/l, made with continual air pumps. After the acclimatization expires (after 5 days) to these fish we have acted with the i/p fixed dose of selected pesticides (300µl, 100µl pesticide dissolved in corn oil and DMSO mixture in the ratio 7:3) which are used more in agriculture, and after 24 hours was done

their dissection and their liver is isolated which then it was handed to the operating procedures with chemicals to determine the EROD enzyme activity, according to the methodology applied by Burke and Mayer (1974) and the enzyme B (a) PMO, the methodology applied by Nebert and Gelboin (1968).

The microsomal fractional preparation for the determination of the EROD enzyme activity

After dissection of the fish, the liver is removed. Liver is removed with care not to hurt the gall bladder (gall bladder is rich in inhibitor OFP). Then it is made the homogenization of the liver with 5 time greater volume of the solution for homogenization (1.15% KCl, 3.6 mM fenilmetilsulfonil fluoride (PMSF) or 0.1 mM pufer of sodium phosphate pH 7.7) in the Potter-Elvehjem homogenizer. The obtained homogenate in this way is centrifuged at 10000g for 20 minutes at 4°C with the centrifuge SANYO Harrier 18/80 REFRIGERATED, to remove the parts of the cell and cell organelles. An upper layer supernatant lipid is carefully removed by vacuum suction, and the supernatant decanted. The decanted supernatant is again centrifuged at 10,000g for 60 minutes. Rotter digested microsomal faction formed in puffer 0.1 M sodium phosphate, pH 7.7 with 20% glycerol and 1g liver used 1ml of this puffer. The entire procedure takes place at +4°C.

Determination of protein

Proteins were determined by using the method of Lowry et al., (1951), and as standard it is used bovine serum albumin (Bovin serum albumin). The amount of proteins was determined by reading the absorbance at 750 nm with Folin's reagent, GENESYS spectrophotometers 10uv.

Determination of the EROD enzyme activity

In fluorimetric quartz plate with optical field is dropped 10mm 1ml Puferit phosphate 0.1M (NaH_2PO_4/Na_2HPO_4) at pH 7.7, NADPH to final concentration of 200µM and substrate (Ethoksyresorufin) to 12.5 µM final concentration. It will be seen an exciting fluorescence wave length emitting 510nm and 585nm in the spectrofluorophotometer SHIMADZU RF-1501, which should be constant. After 1 minute is added the enzymatic preparation (resuspended microsomes) and it is followed the fluorescence increases for over 2 minutes.

After this action, resorufin is added to the plate with (0.585 pm) of the final product of the reaction, and as a result it was immediately noticed the fluorescence increase. The amount of EROD enzyme activity is calculated from the average fluorescence change for 1 minute in in relation to fluorescence change after the addition of the final product.

Results and Discussion

In Table 1 are presented the results obtained from the induction of the EROD enzymes and B (a) PMO fish liver, Barbus peloponnesius by i/p action with 300µl mixture of corn oil and DMSO in a ratio of 3: 7.

From Table 1 it is clear that fish treated with 300 µl mixture of corn oil and DMSO in 3:7 ratio did not show any EROD enxyme activity and B (a) PMO.

Tab. 1 Results obtained from EROD and B (a) PMO enzyme inductivity is checked in the black liver fish, Barbus peloponnesius of the River Vardar during i/p operation with 300µl corn oil mixture and DMSO in a ratio of 3:7.

ERODE inductivity enzymes and B(a)PMO in the experimental black liver fish, (Barbus peloponnesius) of Vardari River		
i/p injection of the dissolution of 300µl of corn oil and DMSO in rapport 3:7	Erode inductivity	B(a)PMO inductivity
	*1,50 ±0,82 (5)**	*1,47±0,70 (5)**

DMSO dimethyl sulfoxide;
± Standard deviation
*Number of fish engaged in the study

Table 2 shows the results obtained from the EROD enzyme induction and B (a) PMO in experimental fish liver Barbus peloponnesius by the i/p action with the solution of 300µl, 100µl pesticide dissolved in corn oil blended with DMSO in the ratio 7:3. From Table 2 it is clear that the EROD enzyme activity and B (a) PMO in the experimental fish increased several times rapidly compared with control fish.

Tab. 2 Results obtained from the EROD enzyme induction and B (a) PMO from the liver fish of Barbus peloponnesius in the River Vardar during i/e action with toxsicants (pesticides, herbicides and fungicides).

Toxicants (pesticides)	ERODE inductivity enzymes and B(a)PMO in the experimental black liver fish, (Barbus peloponnesius) of Vardari River	
	ERODE inductivity	B(a)PMO inductivity
PYRINEX 48 EC[1]	91,15 ± 32,37 (8)*	37,96 ± 23,14 (4)*
CHLOMOREL D[1]	75,50 ± 10,60 (3)*	29,16 ± 13,23 (5)*
STOMP 330[2]	1,65 ± 0,80 (4)*	1,54 ± 0,72 (5)*
LINUREX 50 SC[2]	1,59 ± 0,65 (5)*	1,50 ± 0,59 (5)*
TILT 250[3]	29,77 ± 22,93 (4)*	18,34 ± 9,18 (2)*
TOPAS[3]	27,78 ± 12,83 (5)*	17,50 ± 3,06 (3)*

i/p intraperitoneal; [3]Fungicid;
DMSO dimethyl sulfoxide; ± standard deviation;
[1]Insecticid; *number of fish engaged in the study
[2]Herbicid.

Based on the referent values obtained from the determination of EROD enzyme activity and B (a) PMO of the liver fish, it can be clearly noticed that in the experimental fish the action with toxicants, such as (pesticides, herbicides and fungicides) has caused the inductivity of such biochemical markers.

The largest inductivity to this action has been observed in EROD enzymes, whereas in the B (a) PMO enzyme the inductivity was smaller. Unlike CHLOMOREL insecticide D and insecticide PYRINEX 48 EC that are shown as the most powerful indicator of these two enzymes, the herbicides used in this study, Stomp 330 and LINUREX 50 SC have not caused inductivity. As insecticides also fungicides are characterized with inductive properties, since the two fungicides included in this study TILT 250 and TOPAS have caused such an increased activity of these two enzymes. These results are compatible with the results obtained from Campbel et al., 1983, Delescluse et al., 1998.

Conclusion

Based on the results obtained from this study we can conclude that in addition to the polycyclic aromatic hydrocarbons and chlorinated pesticides, may influence to the increase of the EROD and B (a) PMO enzymes activity. Insecticides and fungicides have caused the increased activity of these two enzymes in contrast to herbicides that haven't showed any significant induction.

Therefore, based on the results obtained, for the ability that possess these kinds of biochemical markers that have to be inducted with *in vitro* conditions, after the action with toxicants (pesticides, herbicides and fungicides), the same study could be carried out even under in vivo conditions, to such species that make the natural population of the Barbus peloponnesius fish in the Vardar River. Results obtained in this study will provide information on the extent of this aquatic ecosystem pollution by pesticides, herbicides and fungicides used in agriculture. It is recommended that pesticides, herbicides and fungicides are not used without control in agriculture because it can lead to lethal consequences for aquatic organisms in general and in particular for fish.

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