Determination of the modulatory potential of atrazine on selected functions of immune cells isolated from rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Atrazine is a widely used compound in major food crops in the world. Therefore, humans and animals are at risk of exposure to atrazine. The purpose of this study was to determine whether different concentrations of atrazine affect the immune cell functions of fish. In the study we determined the proliferative ability of T and B lymphocytes. The phagocytes were assayed for extracellular production of reactive oxygen species in the phagocytic respiratory burst. In the in vitro study fish were intoxicated with atrazine at doses of 1, 2.5, 5, 10 µg/ml. The results have shown the modulatory effects of atrazine on the selected functions of immune cells isolated from blood and head kidney of rainbow trout.

Key words: *atrazine, metabolic activity of phagocytes, lymphocyte proliferation.*

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Introduction

After reviewing the existing information dealing with toxic pesticides it is evident that humans and animals have suffered adverse health consequences from exposure to this chemicals. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine, ATR) is a member of the chlorotriazine family. It is widely used in controlling both grasses and weeds in many crops and in non-agricultural situations such as railways, highways and industrial sites. It is one of the most common pesticide contaminant detected in rain, ground waters, rivers, streams and wells, due to its considerable persistence and mobility in soil and water [1-4].

Fish are particularly sensitive to chemical exposure, especially during early development. Identification of immunological biomarkers in fish would improve their use as model animals for biomonitoring of the aquaculture. Atrazine has a low level of bioaccumulation in fish. It is metabolised in the kidneys and then excreted by the gills [5]. This chemical compound has been found to affect a variety of physiological processes in fish, causing damage of the gill epithelium [6], including disarrangements of osmoregulation, increased respiration, decreased reflexes, and inhibition of acetylcholinesterase in blood serum and brain [7, 8]. Atrazine has been shown to affect metabolism [9, 10] and behaviour of goldfish [11, 12]. Furthermore, exposure to low environmental levels of this herbicide induces a toxic effect on the olfactory system of the salmon [13].

The aim of this study was to investigate the *in vitro* effects of atrazine on the selected immune functions of cells isolated from rainbow trout (*Oncorhynchus mykiss*).

Materials and methods

Reagent

Pure pesticide standard was purchased from Riedel – deHaën, (Sigma Aldrich). Atrazine was first dissolved in DMSO – dimethylsulfoxide (POCh, Lublin, Poland) then diluted in PBS. Dilutions were prepared in such a way that

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the final concentration of DMSO in all conditions did not exceed 0.01%. Solutions were sterilised by filtration.

Fish

The study was carried out on cells isolated from blood (caudal vein) and head kidney of rainbow trout weighing 300-350 g. Healthy animals were obtained from a commercial farm. Experimental animals were treated according to the Local Committee of Ethics (approval number 492/2004).

Separation of leukocytes from fish

Phagocytes and leukocytes were isolated from blood and pronephros. Blood was collected from the caudal vein and diluted 1:2 in RPMI 1640 medium without Ca^{2+}/Mg^{2+} (Biomed, Lublin, Poland). Before separation of organs, fish were anaesthetised with Propiscin (IRS, Żabieniec, Poland) at a concentration of 2 ml/L. Rainbow trout head kidney was removed aseptically and pushed through a 60 µm nylon mesh with RPMI 1640 medium (Biomed, Lublin, Poland). The selected populations of cells were separated by centrifuging method in the appropriate gradient solution.

Lymphocyte proliferative activity assay

Lymphocytes were separated by centrifugation at 1000 x g for 30 min using Gradisol L (1.077 g/ml, Aqua-Medica, Łódź, Poland) gradient. The cells at the interface were harvested, washed twice in phosphate-buffered saline (PBS, Biomed, Lublin, Poland), and resuspended in RPMI 1640 supplemented with 10% foetal calf serum (FCS, Gibco, UK) and 1% penicillin/streptomycin (Sigma, Aldrich). Viable cells were determined by trypan blue exclusions and were evaluated to be more than 98%. The cells were dispensed into 96-well plates at a concentration of 1 – 3 x 10^6 cells/ml.

The proliferative response of lymphocytes to mitogens was determined using the spectrophotometric method – the MTT [14]. Lymphocyte suspensions (100 µl/well) were placed in microtiter plate. 50 µg/ml of conconavalin A – ConA (Sigma, Aldrich) as a T cell mitogen or 10 µg/ml lipopolysaccharide – LPS (Sigma, Aldrich) as a B cell mitogen were added to the cell suspension. After 2 days of incubation, 20 µl of solution containing 7 mg/ml of MTT (3-[4,5-dimethylthiazol-2 yl]-2,5-diphenyltetrazolium bromide) (Sigma, Aldrich) in PBS was added to each well and the plate was left in an incubator for the next 4 h. The optical density was measured at a wavelength of 630 nm on a plate microreader Stat Fax 2600 (Awarness Technology, Finland).

Respiratory burst activity

Respiratory burst activity of phagocytic cells was measured by the reduction of nitroblue tetrazolium (NBT, Sigma, Aldrich) by intracellural superoxide radicals produced by leucocytes stimulated with PMA – phorbol myristate acetate (PMA, Sigma, Aldrich) as described for rainbow trout by Chung and Secombes [15].

The diluted blood and the cell suspension from head kidney were placed separately on a density gradient-Gradisol G (1.115 g/ml, Aqua-Medica, Poland), then centrifuged (1000 x g, 40 min, 10°C). The obtained cells were washed twice in PBS without Ca^{2+}/Mg^{2+} and suspended in the RPMI with 0.1% FCS. Viable cells were determined by trypan blue exclusions and were evaluated to be greater than 95%. Phagocytic cells (mainly neutrophils) were adjusted to the concentration of 3-5 x 10^6 cells/ml. Cells were dispensed in 96-well plates. After 2 h at 18°C , unattached non-adherent cells were washed off and 100 µl of 0.1% NBT solution in RPMI 1640 without phenol red with 1 µg/ml PMA was added to each well. The mixture was incubated for 30 min at 18°C and then supernatant was removed and the adherent cells were fixed with ethanol. Plates were left to dry. The formazan in each well was dissolved in 120 µl of 2MKOH (POCh, Lublin, Poland) and 140 µl of dimethylsulphoxide and the

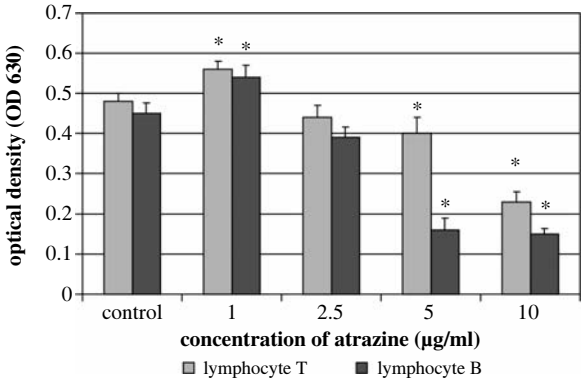

Fig. 1. *In vitro* effects of atrazine on proliferative ability of lymphocytes T and B isolated from head kidney. Data are shown as mean ±SD, n=10, * – differences statistically significant

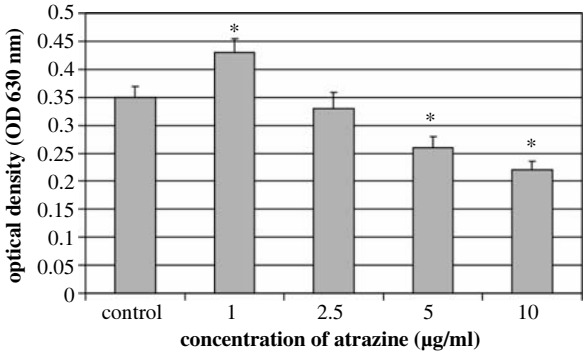

Fig. 2. *In vitro* effects of atrazine on metabolic activity of phagocytes (RBA) isolated from head kidney. Data are shown as mean ±SD, n=10, * – differences statistically significant

Table 1. *In vitro* effects of atrazine at doses of 1, 2.5, 5, 10 µg/ml on metabolic activity (RBA) of blood phagocytes and lymphocyte proliferation stimulated by Con A or LPS in rainbow trout. Data are shown as mean ±SD, n=10. Statistical differences ($P<0.05$) between groups are indicated by *

Parameters	Concentration of atrazine µg/ml medium				
	control	1	2.5	5	10
Proliferative ability of lymphocytes T (ConA)	0.32±0.03	0.36±0.05	0.30±0.02	0.23±0.04*	0.15±0.07*
Proliferative ability of lymphocytes B (LPS)	0.35±0.04	0.39±0.02	0.34±0.05	0.26±0.03*	0.12±0.08*
Respiratory burst activity (RBA)	0.26±0.06	0.28±0.03	0.21±0.04*	0.17±0.03*	0.15±0.07*

optical density was measured by a plate microreader Stat Fax 2600 at 630 nm.

Statistics

All data were treated statistically using one – way analysis of variance (ANOVA). Duncan's post-hoc test was used to determinate differences between groups. In all cases, significance was accepted if $P<0.05$.

Results

Table 1 shows the *in vitro* effects of atrazine on the selected immune parameters of the cells isolated from blood in rainbow trout (respiratory burst activity – RBA, lymphocytes proliferation stimulated by ConA or LPS). The addition of atrazine at concentrations of 5 and 10 µg/ml medium to the immune cells suspension decreased the measured parameters. A slight increase of the mitogenic response of lymphocytes and the metabolic activity of phagocytes were seen at a concentration of 1 µg/ml atrazine.

Figure 1 and figure 2 show the results referring to cells isolated from head kidney. The difference of activity between the cells isolated from blood and cells isolated from kidney was not significant. Proliferative activity of T and B lymphocytes (figure 1) and metabolic activity of phagocytes (figure 2) was significantly reduced after intoxication by atrazine at the concentrations between 5 and 10 µg/ml medium. After administration of atrazine at the lowest concentration (1 µg/ml medium) stimulation of lymphocyte and phagocyte activity was noted.

Discussion

A number of recent studies reported in the literature suggests that triazines are endocrine disrupters and may be cancerogenic [16-19]. Both the toxicity of pesticides and the immunotoxicity of those compounds to organisms should be considered in risk assessment of pesticides. The immune system can be affected by xenobiotics, and these effects can involve the modulation, stimulation or suppression of immune responses.

The data on the immune effects of atrazine are rather controversial and reported different results depending on the test system or organism used in the experiments. Some studies performed on higher vertebrates have shown that atrazine can disrupt normal immune system functioning, enhancing the risk of infectious diseases or cancer [20, 21]. On the other hand, the studies conducted by Fournier et al. [22] and Porter et al. [23], indicate that the herbicide does not suppress antibody production in mice.

Information on the immune effects of atrazine on fish species is limited. The present experiment is a continuation of our previous study, which indicated some negative effects of atrazine on cell mediated and humoral immune response in carp. In this *in vitro* study atrazine at concentrations of 5 and 10 µg/ml of medium has an immunosuppressive effect, which was marked by a decreased level of antibody producing cells. Furthermore, in the *in vivo* study this herbicide decreased the number of antibody secreting cells (ASC) after application of Furogen vaccine [24]. On the contrary, Cossarini-Dunier et al. [25, 26] did not observe any negative effects of atrazine on cellular immunity and antibody production against *Yersinia ruckeri*.

The suppressive activity of atrazine on phagocyte functions suggests that phagocytic cells might be one of the targets of the immunosuppressive activity of atrazine. These effects are related to stimulation of the nonspecific part of the immune system – through phagocytosis and perhaps by the modulation of cytokine production. Results demonstrated by Kim et al. [27] showed that simasine decreases murine macrophage – mediated NO and TNF-α production. However, simasine had slight effect on phagocytosis and the level of hydrogen peroxide (H_2O_2), interleukin-1 and interleukin-6 by LPS – stimulated macrophages. Mechanisms by which atrazine induces those effects on macrophages are not fully understood.

Our results are in agreement with those achieved by Karrow et al. [28], which have demonstrated the immunological changes depending on atrazine concentrations. Overall, the results of the present study here shown that the used concentrations of atrazine may significantly modulate various functions of the immune system of rainbow trout. Thus, at low concentrations atrazine clearly stimulates the lymphocyte proliferation and the metabolic activity of phagocytes, but at high concentrations this chemical compound diminishes those two immunological parameters.

References

1. Felding G (1992): Leaching of atrazine into groundwater. Pestic Sci 35: 39-41.
2. Solomon KR, Baker DB, Richards RP, et al. (1996): Ecological risk assessment of atrazine in North American surface waters. Environ Toxicol Chem 15: 31-76.
3. Graymore M, Stagnitti F, Allinson G (2001): Impact of atrazine in aquatic ecosystems. Environ Int 26: 483-495.
4. Southwick LM, Grigg BC, Fouss JL, Kornecki TS (2003): Atrazine and metolachlor in surface runoff under typical rainfall conditions in southern Louisiana. J Agric Food Chem 51: 5355-5361.
5. Fischer-Scherl T, Vesser A, Hoffmann RW, et al. (1991): Morphological effects of acute and chronic atrazine exposure in rainbow trout (Oncorhynchus mykiss). Arch Environ Contam Toxicol 20: 454-461.
6. Alzemi BM, Lewis JW, Andrews EB (1996): Gill damage in freshwater fish Gnathonemus petersii (family: Mormyridae) exposed to selected pollutants: An ultrastructural study. Environ Technol 17: 225-238.
7. Gluth G, Hanke W (1985): A comparison of physiological changes in carp, Cyprinus carpio, induced by several pollutants at sublethal concentrations. I. The dependency on exposure time. Ecotoxicol Environ Saf 9: 179-188.
8. Hussein SY, El-Nasser MA, Ahmed SM (1996): Comparative studies on the effects of herbicide atrazine on freshwater fish Oreochromis niloticus and Chrysichthyes auratus at Assiut, Egypt. Bull Environ Contam Toxicol 57: 503-510.
9. Grobler E, van Vuren JJJ, du Preez HH (1989): Routine oxygen consumption of Tilapia sparrmanii (Cichlidae) following acute exposure to atrazine. Comp Biochem Physiol 93C: 37-42.
10. Srinivas T, Prasad TA, Raffi GM, Reddy DC (1991): Effect of atrazine on some aspects of lipid metabolism in fresh water fish. Biochem Int 23: 603-609.
11. Saglio P, Trijasse S (1998): Behavioral responses to atrazine and diuron in goldfish. Arch Environ Contam Toxicol 35: 484-491.
12. Steinberg CEW, Lorenz R, Spieser OH (1995): Effects of atrazine on swimming behavior of zebrafish, Brachydanio rerio. Wat Res 29: 981-985.
13. Moore A, Lower N (2001): The impact of two pesticides on olfactory-mediated endocrine function in mature male Atlantic salmon (Salmo salar L.) parr. Comp Biochem Physiol B Biochem Mol Biol 129: 269-276.
14. Mosmann T (1983): Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J Immunol Methods 65: 55-63.
15. Chung S, Secombes CJ (1988): Analysis of events occurring within teleost macrophages during the respiratory burst. Comp Biochem Physiol 89b: 539-544.
16. Biradar DP, Rayburn AL (1995): Flow cytogenetic analysis of whole cell clastogenicity of herbicides founded in groundwater. Arch Environ Contam Toxicol 28: 13-17.
17. Kettles MA, Browning SR, Prince SW, et al. (1997): Triazine herbicide exposure and breast cancer incidence: an ecologic study of Kentucky countries. Environ Health Perspect 104: 1324-1331.
18. Cooper RL, Stoker TE, Tyrey L, et al. (2000): Atrazine disrupts the hypothalamic control of pituitary-ovarian function. Toxicol Sci 53: 297-307.
19. Spano L, Tyler CR, Van Aerle R, et al. (2004): Effects of atrazine on sex steroid dynamics, plasma vitellogenin concentration and gonad development in adult goldfish (Carassius auratus). Aquat Toxicol 66: 369-379.
20. Böcher M, Böldicke T, Sasse F (1993): Cytotoxic effects of atrazine on murine B - lymphocytes in vitro. Sci Tot Environ 132: 429-433.
21. Hooghe RJ, Devos S, Hooghe-Peters EL (2000): Effects of selected herbicides on cytokine production in vitro. Life Sci 66: 2519-2525.
22. Fournier M, Friborg J, Girard D, et al. (1992): Limited immunotoxic potential of technical formulation of the herbicide atrazine (Aatrex) in mice. Toxicol Lett 60: 263-274.
23. Porter WP, Jaeger JW, Carlson IH (1999): Endocrine, immune, and behavioral effects of aldicarb (carbamate), atrazine (triazine), and nitrate (fertilizer) mixtures at groundwater concentrations. Toxicol Ind Health 15: 133-150.
24. Rymuszka A, Siwicki AK, Studnicka M, et al. (2002): In vitro and in vivo studies on the effect of selected pesticides on the number of antibody secreting cells (ASC) and specific immune response in carp (Cyprinus carpio L.). Acta Pol Toxicol 2: 181-189.
25. Cossarini-Dunier M (1987): Effects of the pesticides atrazine and lindane and the manganese ions on cellular immunity of carp, Cyprinus carpio. J Fish Biol 31(Suppl A): 67-73.
26. Cossarini-Dunier M, Demael A, Riviere JL, et al. (1988): Effects of oral dose of the herbicide atrazine on carp (Cyprinus carpio). Ambio 6: 401-405.
27. Kim KR, Son EW, Rhee DK, Pyo S (2002): The immuno-modulatory effects of the herbicide simazine on murine macrophage functions in vitro. Toxicol In Vitro 16: 517-523.
28. Karrow NA, McCay JA, Brown RD, et al. (2005): Oral exposure to atrazine modulates cell-mediated immune function and decreases host resistance to the B16F10 tumor model in female B6C3F1 mice. Toxicology 209: 15-28.