ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Aluminium induced cholinotoxicity in zebra fish brain - A sequel of oxidative stress

***S.L.Maheswari [1], R.Venkatakrishna Murali , and R.Balaji**

Director, National Centre for Neurotoxicity Research to Assist Drug Development, Department of Pharmacology and Environmental Toxicology, Dr.ALM. Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani,

Manuscript Info

Manuscript History:

Received: 16 December 2013
Final Accepted: 27 January 2014
Published Online: February 2014

Key words:
Aluminium, zebrafish, Acetylcholinesterase (AChE),Oxidative stress, Antioxidants.

****Corresponding Author***

S.L.Maheswari

Abstract

Aluminium has been reported to have biological effects that make the organism's health in jeopardy when the exposure exceeds the adaptive capability. Exposure to aluminium chloride for 21 days has been found to alter Acetylcholinesterase (AChE) activity and oxidative status in brain tissue of zebra fish in the present study. When groups of zebrafish were exposed to non-lethal concentration of $AlCl_3$, there was an increasing trend in the activity of the enzyme AChE up to 14 days. As the enzyme is vital in the hydrolysis of the neurotransmitter Acetylcholine (ACh) which plays an important role not only in neurotransmission but also in the behaviour of the animal, this observation can have a serious implication on the survival of the animal. The oxidative stress assessed by Lipid peroxidation (LPO) is also found to be increased. The response of tissue antioxidants assessed by glutathione s-transferase (GST), Catalase and reduced glutathione (GSH) varied in animals exposed to $AlCl_3$ for different durations. While some of the responses of the antioxidants correlate with the extent of free radicals produced as assessed by LPO, the importance of correlating this response to alterations in the AChE has been discussed.

Introduction

Cholinergic functions of the central nervous system (CNS) mainly depend on the neurotransmitter acetylcholine (ACh). After release from presynaptic nerve terminals, acetylcholine (ACh) is rapidly removed from the synaptic cleft by acetylcholinesterase, which belongs to the family of type B carboxylesterases and cleaves acetylcholine into choline and acetate (Soreq and Seidman, 2001). Any change in the activity of this enzyme reflects altered cholinergic function. Aluminium has been reported to cause changes in cholinergic function of the CNS. It has been suggested that aluminum interacts with the cholinergic system, acting as a cholinotoxin (Gulya et al., 1990). Alteration in the cholinergic function of CNS has also been reported to cause behavioral changes which can be detrimental to the survival of the organism. Increasing domestic as well as industrial use of aluminium due to its low cost and ubiquitous presence with consequent augmented discharge in to water bodies has increased the exposure of aquatic organism to this metal in quantities greater than before. Aluminium in acidic environments presents severe risks to the aquatic biota, including fish (Waring et al., 1996). Zebrafish (*Danio rerio*) has been recognized as a toxicological model for chemical toxicity (Hill et al., 2005) and it has been shown that the cholinergic system is widely distributed in the zebra fish brain (Park et al., 2008). AChE is the only ACh-hydrolyzing enzyme in zebra fish (Behra et al., 2002) and measurement of AChE activity in organisms is widely used as a specific biomarker of toxicity (Roex et al., 2003). The present investigation envisages assessing the consequences of exposure to aluminium in zebra fish with reference to alteration in AChE activity as well as oxidative status in brain tissue.

MATERIALS AND METHODS

ANIMALS

Adult, healthy, wild-type Zebra fish of both sexes got from licensed supplier were acclimatized for three weeks in 20 litre aquarium tank with aerated water maintained at a temperature of 26±2^{o}C , 14 hr/10hr light/dark cycle photo period and fed twice a day with fish feed.

CHEMICALS

All the chemicals used were of analytical grade. Aluminium chloride (SRL, India), Thiobarbituric acid and Malondialdehyde got from Sigma-Aldrich corp. were used.

TREATMENT

After acclimatization period the animals were divided into four groups each having six animals. Each group was maintained in separate aerated tanks and water was changed every 24 hrs. The test groups were exposed to $AlCl_3$ 150ppm, the concentration being arrived at based on previous studies and the duration of study was 7 days (short duration), 14 days (moderate duration) 21 days (longer duration). The control group was maintained in the same conditions but without $AlCl_3$ for 21 days.

SAMPLE PREPARATION

At the end of each exposure period, each animal was cryo-anesthetized, brain dissected out without any damage after decapitation, washed with saline, weighed, homogenized in microfuge tube by plastic micro homogenizer using 0.5 ml of ice cold phosphate buffer (pH 8) for AChE, sucrose (0.25M) for LPO, 0.1M PO_4 buffer (pH 7.0) for catalase, 50 mM phosphate buffer (pH 7.0) with 1mM EDTA for GST and distilled water for GSH determination. This was centrifuged and the supernatant used as sample source.

DETERMINATION OF AChE ACTIVITY

AChE activity was analysed based on the procedure developed by Orhan et al. (2011) from the method of Ellman et al. (1961) with suitable modification. Essentially 0.1 mM sodium phosphate buffer (pH 8.0), DTNB, and samples added in a 96-well microplate was incubated for 15 min at 25 °C and the reaction initiated by the addition of acetylthiocholine iodide as per the procedure. Phosphate buffer served as blank and eserine sulphate was used as the inhibitor for specific acetylcholine esterase activity. The reaction was monitored by microplate reader (Beckman Coulter Multimode Detector, DTX-880) at 412 nm for 5 minutes and rate of enzyme activity was calculated.

ESTIMATION OF PROTEIN: Tissue protein was assayed by following the procedure of Lowry et al., (1951)

LIPID PEROXIDATION

Assessment of lipid peroxidation was done as per the procedure of Ohkawa et al., (1979). The mixture of sample, SDS (8.1%) and acetic acid (pH 3.5) was added with thiobarbituric acid (0.8%). After incubation at 95^{0}C for 60 minutes, 1.0ml distilled water was added, absorbance noted at 532nm by Shimadzu UV-Vis Spectrophotometer model UV 2450, and concentration read from standard graph for absorbance against known concentrations of Malondialdehyde in 0.1N HCl.

CATALASE ACTIVITY

Catalase activity was assayed as described by Sinha (1972). Reaction of the mixture containing 0.4ml 0.01M PO_4 buffer (pH 7.0), 0.1ml sample and 0.5ml 0.2M hydrogen peroxide was arrested by addition of 2.0 ml dichromate acetic acid reagent (1:3 5% $K_2Cr_2O_7$ and glacial acetic acid) at 0,30 and 60 sec interval and kept in boiling water bath for 10 minutes, cooled and read at 570nm by Spectrophotometer. For blank instead of the reaction mixture 1.0ml of PO_4 buffer was used. Enzyme activity was read from standard graph constructed with known concentrations of hydrogen peroxide.

GLUTATHIONE S-TRANSFERASE

GST activity was assayed spectrophotometrically as per the method of Habig et al. (1974). To 0.1ml 1mM GSH and 1.0ml PO_4 buffer, 0.5ml sample and 1.3ml of distilled water for test or distilled water 1.8ml alone for blank was added. Reaction was started by adding 0.1ml 30 mM 1-chloro 2,4-dinitro-benzene (CDNB) after preincubation at room temperature for 2 minutes and increase in activity measured for 3 minutes at 340 nm by Spectrophotometer.

REDUCED GLUTATHIONE ACTIVITY

GSH was determined by the method of Moron et al ,(1979). 0.8 ml of 0.2M PO_4 buffer and 0.2ml of 0.6mM DTNB were added with 0.2ml of 5% TCA for blank and 0.2ml sample (got after protein precipitation with 5%TCA of the homogenate and centrifugation) for test and readings taken at 412nm by spectrophotometer. GSH in the sample was calculated from a standard graph constructed with known concentrations of GSH.

STATISTICAL ANALYSIS

Data was compiled and the values were expressed as mean ± SD. Two way Analysis of Variance (ANOVA) followed by Student- Newman- Keuls pair-wise multiple comparison was done by Sigma Stat 3.5 software. Difference between the mean values with $p<0.05$ was considered as statistically significant.

RESULTS

There was no much fluctuation in the AChE activity in the brain tissue of control animals throughout the experimental period. Compared to the control group, increase in AChE activity was seen in the groups exposed to $AlCl_3$ for 7 days ($p<0.01$) and 14 days ($p<0.001$).Increase in enzyme activity due to $AlCl_3$ exposure was more after 14 days than after 7 days ($p<0.001$). Though the enzyme activity was more in animals exposed $AlCl_3$ for 21 days than the control group, it was significantly lesser ($p<0.001$) when compared with the activity seen in the group exposed to $AlCl_3$ for 14 days (Fig.1).

Exposure to aluminium for 7 days did not alter the tissue protein content (Fig.2). Tissue protein in $AlCl_3$ exposed group after 14 days was higher than the control group ($p<0.001$) and also greater than that seen after exposure for 7 days ($p<0.001$). Protein content was lesser in the group exposed to $AlCl_3$ for 21 days than the control group as well as groups exposed $AlCl_3$ for 7 and 14 days ($p<0.001$)

Compared to the control animals, lipid peroxidation was more in the animals treated with $AlCl_3$ for 7 ($p<0.01$), 14 and 21 days ($p<0.001$). LPO recorded in animals treated with $AlCl_3$ for 14 days and 21 days was lesser ($p<0.01$) than those treated for 7 days (Fig.3).

Catalase activity was not altered in the groups exposed for 7 and 14 days (Fig.4), while an increase was observed

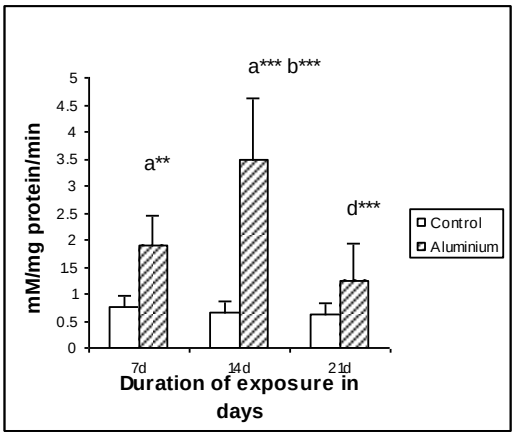

Fig.1. Effect of Aluminium on Brain AChE

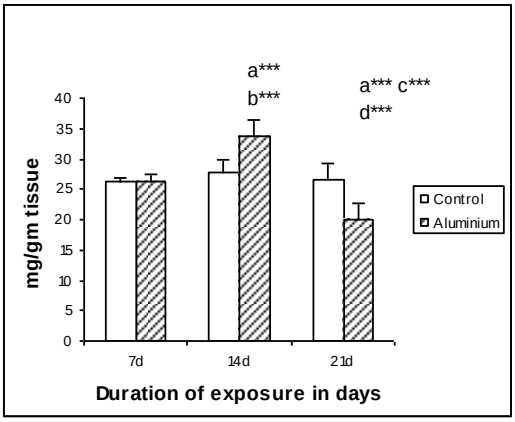

Fig.2. Effect of Aluminium on Protein

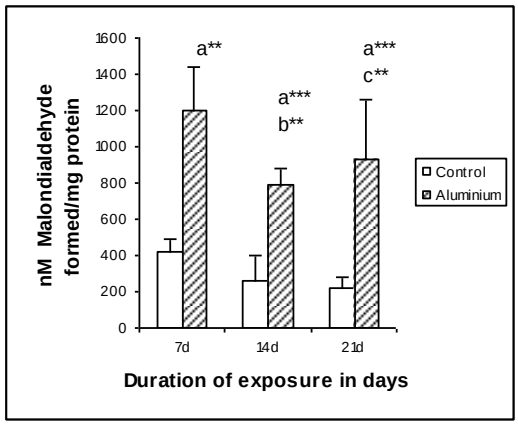

Fig.3. Effect of Aluminium on LPO

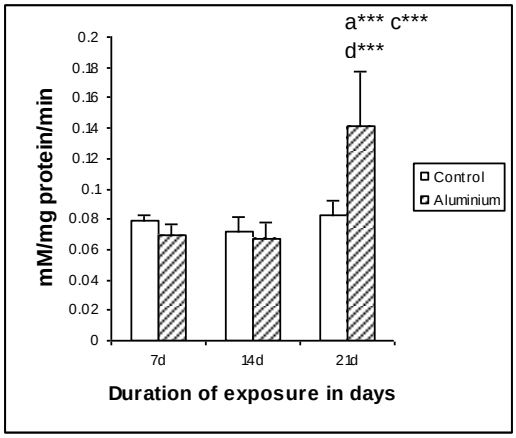

Fig.4. Effect of Aluminium on Catalase

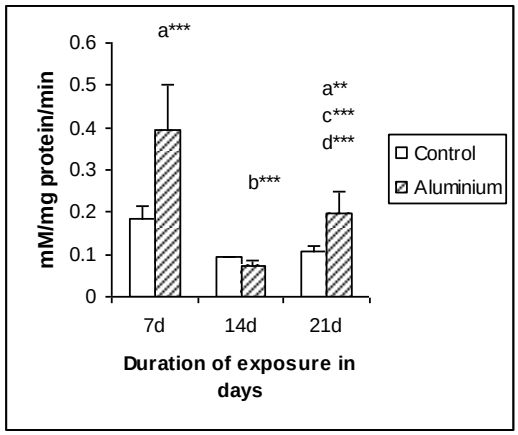

Fig.5. Effect of Aluminium on GST

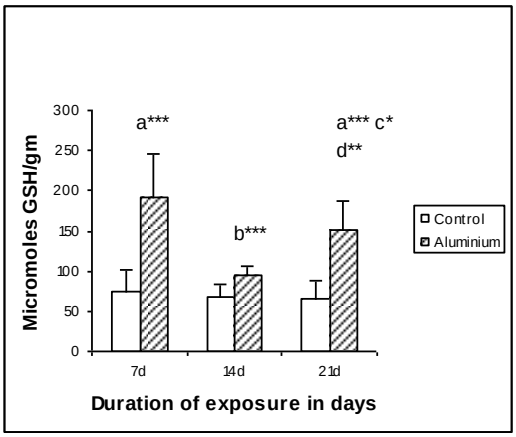

Fig.6. Effect of Aluminium on GSH

n=6 * p<0.05 ** p<0.01 *** p<0.001
a=Control vs $AlCl_3$ b=7days vs 14 days
c=7days vs 21days d=14 days vs 21days

in the group exposed for 21 days (p<0.001).Tissue GST was significantly increased in animals exposed for 7 days (p<0.001) as well as 21 days (p<0.01) when compared with corresponding control groups (Fig.5). Compared to animals exposed for 7 days, GST activity was lesser in animals exposed for 14 and 21 days (p<0.001). However the activity of the enzyme in the group exposed for 21 days was more than that seen with the group exposed for 14 days (p<0.001).

Increase in GSH (p<0.001) was seen in the groups exposed to $AlCl_3$ for 7 and 21 days (Fig.6). Compared to the group exposed to $AlCl_3$ for 7 days, GSH in the groups exposed for 14 days (p<0.001) and 21 days was lesser (p<0.05). GSH was more in the group exposed for 21 days compared to the group exposed for 14 days (p<0.01).

DISCUSSION

It has been suggested that aluminum interacts with the cholinergic system, acting as a cholinotoxin (Gulya et al., 1990). AChE in fish is mainly cholinergic and its activity is essential for normal behaviour and muscular function (Kirby et al., 2000). Interaction of aluminium with cholinergic system has been observed as brain AChE alterations in zebrafish exposed to $AlCl_3$ accompanied by alteration in tissue protein as well as other parameters

included to assess the oxidative status in the present study. The initial increase in brain AChE activity for short duration seems to be the influence of aluminium on the enzyme activity rather than the quantity of the enzyme available, as the protein content during the corresponding period of assessment do not correlate with the changes in activity of the enzyme. However, enhanced AChE activity accompanied by higher protein content after exposure for moderate duration indicates the increased tissue content of the enzyme. Increase in brain AChE activity due to aluminum treatment has been reported by Kaizer et al. (2005) in mice. Senger et al., (2011) have shown that aluminum treatment, at acid pH, causes changes in brain AChE activity and behavioral parameters in zebra fish and suggested that induction of brain AChE activity could be involved in the behavioral and neurotoxic effects of aluminum on the central nervous system. This effect of aluminium seems to wean off during further exposure as there was a decreasing trend in the activity of enzyme with corresponding decrease in protein content. The mechanisms involved to counter this effect of the metal seem to become effective during the later phase of exposure as there was a decrease in the trend compared to the initial phase.

Though there was no change in the tissue protein content initially, an increase was seen after exposure for 14 days. As there was an increased AChE activity, the increase in protein content could be attributed to enhanced enzyme production. While the increase can be attributed to increased production of enzymes or stress caused by exposure to the metal, the later fall in tissue protein reflects the possible adverse impact during continued exposure. Thus shorter duration of exposure to aluminium has been found to have no influence on tissue protein whereas continuous exposure for longer duration causes stress followed by decrease in tissue protein.

Brain tissue rich in polyunsaturated lipids is highly vulnerable to ROS mediated oxidative damage (Siegel et al., 1999) and increase in LPO was observed during all durations of exposure to aluminium. Increased generation of reactive oxygen species (ROS) is implicated in the pathogenesis of many diseases and in the toxicity of a wide range of compounds (Halliwell and Gutteridge, 1985).Though the extent of this effect was comparatively lesser during moderate duration of exposure, the mechanisms involved in counteracting the free radical production during repeated exposure seems to be inadequate as increase in LPO was more in animals exposed for longer duration studied. Oxidative stress has been reported to be an important factor in the pathogenesis of many abnormalities, and alterations observed in AChE activity in this study could be due to increased free radical production during aluminium exposure. As many metals crossing the blood brain barrier can accumulate in brain, promoting the generation of oxidative stress and alterations in the metabolism of some proteins associated with the development of neurodegenerative diseases (Richetti et al., 2011), altered cholinergic functions expressed as changes in AChE activity in this study could be a sequel to oxidative stress caused by aluminium.

GST activity has been found to be increased during shorter as well as longer duration of exposure to aluminum. The extent of activity seems to be more during initial stages of exposure. Though there was decrease in the enzyme activity after moderate duration of exposure, the increase in the GST activity in the group exposed for longer duration studied indicates continuous exposure can result in enhancement of the enzyme activity. GSTs play a vital role in protecting tissues against oxidative damage and oxidative stress. (Vontas et al., 2001).Thus GST activity seems to be enhanced in response to the increased free radical production as the extent of the enzyme activity corresponds to extent of LPO recorded in animals exposed to aluminium for different durations.

Unlike GST activity, enhancement in tissue catalase activity becomes conspicuous only during prolonged exposure to aluminium for a longer duration. This could be the result of demand for more tissue antioxidants since the increase in other antioxidants is not adequate enough to scavenge the quantum of free radicals produced during longer duration of exposure. Reduced glutathione has been found to follow the pattern of LPO. Thus GST and GSH seem to be the more specific tissue antioxidants involved in scavenging free radical production during aluminium exposure.

It is to be noted that brain cells have relatively low antioxidant defenses (Ward et al., 1994) and hence despite the enhancement in tissue enzymatic and non enzymatic antioxidants, LPO was found to be more in the group exposed for longer duration. Thus free radical production is found to exceed the scavenging capability of tissue antioxidants during long term exposure to aluminium. Because of the low antioxidant defenses, brain cells can be susceptible to metal toxicity (Crichton et al., 2002) and aluminium induced changes in AChE indicative of a cholinotoxic impact could be due to oxidative stress.

In conclusion, zebrafish exposed to aluminium showed increase in free radical production accompanied by increased brain AChE activity indicating the possible cholinergic dysfunction. An increase in tissue antioxidants during exposure to aluminium indicates the body mechanisms involved in scavenging the free radicals produced in excess coming in to play. Induction of the tissue antioxidants is found to be inadequate to counter the extent of free radicals produced during long-term exposure to aluminum indicating the cholinotoxic potential of the metal as a consequence of oxidative stress.

ACKNOWLEDGEMENT

This study was carried out in the National Centre for Neurotoxicity Research, University of Madras funded by the Department of Science and Technology (DST-DPRP). The authors thank DST-DPRP, Ministry of Science and Technology for the funding to establish the National Facility and the encouragement given to carry out neurotoxicological research.

REFERENCES

1. Behra M., Cousin X., Bertrand C., Vonesch J.L., Biellmann D., Chatonnet.A.(2002).Acetylcholin-esterase is required for neuronal and muscular development in the zebrafish embryo. Nat Neurosci. 5(2):111–8.
2. Crichton R.R., Wilmet S., Legssyer R., Ward R.J. (2002) Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells. J Inorg Biochem 91:9–18
3. Ellman G.L., Courtney, K.D., Andres, V.J.R.., and Featherstone, R.M. (1961) "A new and rapid colorimetric determination of acetylcholinesterase activity". Biochemical Pharmacology, 7:88-95.
4. Gulya K., Rakonczay Z., Kasa P. (1990) Cholinotoxic effects of aluminum in rat brain. J Neurochem, 54:1020–6.
5. Habig W.H., Pabst M.J., and Jakoby W.B. (1974). Glutathione S-Transferases The first enzymatic step in mercapturic acid formation. J.Biol.Chem., 249: 7130-7139..
6. Halliwell B. and Gutteridge J.M.C. (1985) Oxygen radicals and the nervous system. Trends Neurosci., 8:22-6.
7. Hill A.J., Teraoka H., Heideman W., and Peterson R. E. (2005) Zebrafish as a Model Vertebrate for Investigating Chemical Toxicity. Toxicological Sciences 86(1): 6–19.
8. Kaizer R.R., Correa M.C., Spanevello R.M., Morsch V.W., Mazzanti C.M., Goncalves J.F., Schetinger M.R. (2005) Acetylcholinesterase activation and enhanced lipid peroxidation after long-term exposure to low levels of aluminum on different mouse brain regions. J Inorg Biochem., 99:1865–1870.
9. Kirby, M.F., Morris, S., Hurst, M., Kirby S.J., Neall P., Tylor T. and Fagg A. (2000) The use of cholinesterase activity in flounder (*Platichthys flesus*) muscle tissue as a biomarker of neurotoxic contamination in UK estuaries. Mar. Pollut. Bull., 40: 780–791.
10. Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193 (1): 265–75
11. Moron M. S., De Pierre J. N. and Manervik V., (1979) Levels of glutathione, glutathione reductase and glutathione -S- transferase activities in rat lung and liver. Bio. Chem. Biophys. Acta., 582: 67-68.
12. Ohkawa H, Ohishi N, Yagi K.(1979) Assay for lipid peroxides in animal tissues by Thiobarbituric acid reaction. Anal Biochem., 95: 351-8.
13. Orhan I E, Yilmaz B S., Altun M. L., Saltan G. and Bilge S.. (2011) Anti-Acetylcholinesterase and Antioxidant Appraisal of the Bulb Extracts of Five *Sternbergia* Species. Rec. Nat. Prod. 5:3,193-201.
14. Park E., Lee Y., Kim Y., Lee C. (2008) Cholinergic modulation of neural activity in the telencephalon of the zebrafish. Neurosci Lett., 439:79–83
15. Richetti, S.K. Rosemberg , D.B. Ventura-Lima J., Monserrat J.M. Bogo M.R. , Bonan. C.D., (2011) Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure NeuroToxicology., 32 116–122
16. Roex E.W., Keijzers R., Van Gestel C.A. (2003) Acetylcholinesterase inhibition and increased food consumption rate in the zebrafish, Danio rerio, after chronic exposure to parathion. Aquat Toxicol., 64:451–460
17. Senger M R., Seibt K. J., Ghisleni G.C., Dias R.D., Bogo M.R., and Bonan C.D. (2011) Aluminum exposure alters behavioral parameters and increases acetylcholinesterase activity in zebrafish (Danio rerio) brain. Cell Biol Toxicol., 27:199–205

18. Siegel G.J., Agranoff B.W., Albers R.W., Fisher S.K. and Uhler M.D., eds. (1999) Basic Neurochemistry; Molecular, cellular and medical aspects. 6th ed. Lippincott Williams & Wilkins, Philadelphia. 1023-1120.
19. Sinha A.K. (1972) Colorimetric assay of catalase. Anal Biochem., 47(2):389-94.
20. Soreq H. and Seidman S.(2001) Acetylcholinesterase - new roles for an old actor. Nat Rev Neurosci., 2(4):294–302.
21. Vontas J. G., Small G. J. and Hemingway J. (2001) "Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in Nilaparvata lugens". Biochem. J. 357, 65-72.
22. Ward R.J., Kuhn L.C., Kaldy P., Florence A., Peters T.J., Crichton R.R. (1994) Control of cellular iron homeostasis by iron responsive elements in vivo. Eur J Biochem., 220:927–931
23. Waring C., Brown J., Collins J., Prunet P. (1996) Plasma prolactin, cortisol, and thyroid responses of the brown trout (Salmo trutta) exposed to lethal and sublethal aluminium in acidic soft waters. Gen Comp Endocrinol., 102:377–85.