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Study of Acetylcholinesterase (AChE) gene expression and itsr relation with RNA content in brain of *Duttaphrynus melanostictus*

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Abstract

Amphibians are important to the overall ecosystem balance. The large biomass of these amphibians makes them significant prey for other animals. Currently amphibian populations are declining in a number of geographical locations throughout the world. In most cases, the cause or causes are unknown, but are assumed to result from manmade alterations in the environment. In this study, AChE expression was investigated in different brain areas cerebrum and cerebellum in male and female *Duttaphrynus melanostictus*. On comparing with male and female showed increase in AChE activity in cerebrum and cerebellum of female Duttaphrynus melanostictus .However, no significant change in AChE activity was found in the cerebrum

and cerebellum of same male and female, Thus it appears that sex alters AChE activity in different brain regions (G4 isoform) that may vary in male and female. Sequence analysis revaled that least divergence was found in between male cerebrum and female cerebrum (14.9) and maximum divergence was found in between male cerebellum and female cerebellum (99.1) with control AChE HM998937.1.

(**Key Words:** Acetylcholinesterase; Physiology; Invertebrates; Vertebrates; Cerebrum**)**

Authorship Statement

There are no conflicts of interest amongst the authors regarding the content of article and clinical work of this case.

Introduction:

The global decline in amphibians came to the world's attention in the beginning of last decade and was vigorously debated (1-5). Since that time, surveys of natural populations have become more extensive and the general consensus is that the frog's population is declining. In tune with the global decline of amphibian population even in Patna (25°37'N 85°12'E) where frogs and toads were found in abundance during monsoon of year 2005, it was difficult to find frogs and toads even during the monsoon season probably due to very little rain and very few insects during the lighting festival last year and also due to indiscriminate use of pesticides (6). Pesticides are receiving increased attention as a potential cause of amphibian decline. Amphibians are an important component of the food and are good bio-indicators of environmental pollution due to their susceptibility to chemicals during their freshwater cycles. The effects of environmental pollution together with changes in human activity and climate have contributed to the reduction of amphibian population. However, toxicological research on amphibians has been rather scarce compared with that on other vertebrates.

Acetylcholinesterase (AChE) is a key enzyme in the nervous system. It terminates nerve impulses by catalysing the hydrolysis of neurotransmitter acetylcholine. As a specific molecular target of organophosphate and carbamate pesticides, acetylcholinesterase activity and its inhibition has been early recognized to be a human biological marker of pesticide poisoning. Measurement of AChE inhibition has been increasingly used in the last two decades as a biomarker of effect on nervous system following exposure to organophosphate and carbamate pesticides in occupational and environmental medicine. The success of this biomarker arises from the fact that it meets a number of characteristics necessary for the successful application of a biological response as biomarker in human biomonitoring: the response is easy to measure, it shows a dose-dependent behavior to pollutant exposure, it is sensitive, and it exhibits a link to health adverse effects.

Cholinesterases (ChEs) have been the pioneer biomarkers for assessing exposure to organophosphate (OP) and carbamate (CB) pesticides in wildlife (7). In vertebrates two types of ChEs were identified based on their distinct substrate specificity and inhibitor sensitivity. The acetylcholinesterase (AChE; EC 3.1.1.7) specifically catalyses the hydrolysis of acetylcholine and is subjected to marked inhibition by its own natural substrate. In contrast, butyrylcholinesterase (BChE; EC 3.1.1.8) is capable of degrading a wider range of choline esters and is not inhibited by its substrate (8). Caballero de Castro et al. (1991) suggested that these enzymes showed, in amphibians tadpoles, different rates of recovery, this fact justify measures of different ChEs (AChE for the brain and BChe for tail) (9)

Materials and Methods:

This prospective study was conducted in Anatomy department of a tertiary care teaching medical school in South India. The study was approved by institutional ethical committee. We ensured that study complied with biomedical ethics guidelines for animal experimentation as laid down by Indian council of Medical Research (ICMR). Duttaphrynus melanostictus **male and female** weighing an average were purchased from a local supplier and transported live to the laboratory in aerated tanks. During the acclimatization period, the Duttaphrynus melanostictus were fed daily (Safe feed 7711, Charoen Pokphand Foods PCL, Thailand) weighing about 1% of the body weight, and were then fasted for 24 h before the experiment. They were sacrificed, the brain was rapidly removed, weighed, and dissected for RNA extraction and sequencing the brain was rapidly removed for RNA extraction followed by reverse transcription and fold induction of gene expression between AChE and 28S rRNA genes, and were then analysized by PCR and Gel analysis.

Total RNA isolation:

Total RNA was extracted from the brain of Duttaphrynus melanostictus using RNeasy Mini Kit (QIAGEN GmbH, Germany),according to the manufacturer's instructions. RNA was analyzed in 1% agarose gel, containing ethidiumbromide and visualized with UV light. The 1 Kb DNA ladder plus and 100 bp DNA ladder plus (Fermentas, USA) was used as molecular marker.

AChE cDNA synthesis and Sequence Analysis

Reverse transcription-polymerase chain reaction (RT-PCR): Complementary DNA (cDNA) was synthesized by using First Strand cDNA Synthesis kit for PCR thermo scientific, according to the manufacturer's instructions. PCR amplification used degenerate primers. Primers of AChE gene (F- 5' GACTTCCATCCCTGACAGATAC'3; R-5' CAGTCACCCACTCGCTAATAC' 3) designed in conserved region of chick from GENBANK using CODEHOP program.

For the PCR reaction, 4 Hl of cDNA from each synthesis were added to 7 Hl of '2X PCR master mix' containing 10X PCR buffer, 10 mM dNTP, 25 mM MgCl2, 5 U of Taq DNA polymerase (Fermentas, USA). Twenty HM of each pair of the primers was added, and the final volume was adjusted to 14 Hl with nuclease free water. The

mixtures were denatured at 94^{o}C for 3 min. Thirty five cycles of PCR were carried out, with denaturation at 94^{o}C for 45 sec, annealing at 57^{o}C for 30 sec, and extension at 72^{o}C for 1 min, followed by a final extension period of 5 min. PCR products were analyzed by electrophoresis on 1% agarose gels stained with GelStar Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc.).
PCR products were cloned into the pGEMT plasmid vector (Promega) and sequenced using forward and reverse primers. Sequencing was performed with the Big DyeTM Terminator Cycle Sequencing Ready Kit, version 3.0 (ABI PrismTM, Perkin Elmer) and an ABI 3700 Applied Biosystems Model automated DNA sequencer. Nucleotide sequences of NWS were analyzed by BLASTN to search for similarities, and sequence alignments was performed with CLUSTAL W (Megalign program, DNASTAR Inc., Madison, WI)

Quantitative assessment of RNA by methyl green-pyronin staining
The tissues are fixed in methacorn fixatives for 4 hours for fixation, and then followed by routine histological processing. The 5 to 6 um sections are taken for all tissues and stained with **MGP (**methyl green pyronin**).** The Number of RNA granules are estimated by image analysis by used software **IMAGE pro 6.2.**

Results:
AChE cDNA sequence
AChE cDNA sequence was used to investigate difference in male and female cerebrum and cerebellum. The ORF of AChE is comprised of 221 nucleotides (GenBank accession number HM998937.1), showing significant nucleotide similarity 22.2% and 99.1% respectively with Duttaphrynus melanostictus male cerebrum and cerebellum whereas 14.9 % and 89.6 respectively with female cerebrum and cerebellum AChE. The highly divergent regions between the AChE sequences when compare with standard sequence are found in male and female cereberum (350) where as when compare with male cerebellum, female cerebellum was less significant, But when compare with male and female cerebrum and cerebellum the highest divergence was found in between male cerebrum and female cereberum (350) and least divergence was found in between male cerebellum and female cerebellum (0.0) (Fig 3)

Expression of AChE gene
Expression of Acetylcholinesterase (AChE) was significantly more in female cerebrum and cerebellum when compare with male cerebrum and cerebellum (Fig.4). Also we found that Methyl green-pyronin staining on Duttaphrynus melanostictus brain regions shows more RNA granules in female cerebrum and female cerebellum (Fig 5).

Figure 1: Alignment of nucleotide sequences of *Duttaphrynus melanostictus* AChE male and female cerebrum and cerebellum with AChE HM998937.1 Boxes residue differ from the consensus

Figure 2: Alignment of peptide sequences of *Duttaphrynus melanostictus* AChE male and female cerebrum and cerebellum with AChE HM998937.1 Boxes residue differ from the consensus

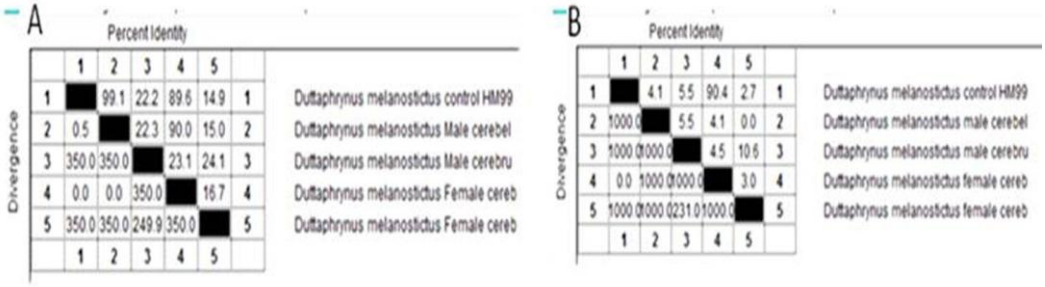

Figure 3: Percentage identity and divergence of *Duttaphrynus melanostictus* AChE NM_2054.18.1 nucleotide and amino acid sequences from and Gallus gallus domesticus AChE of male and female cerebrum and cerebellum

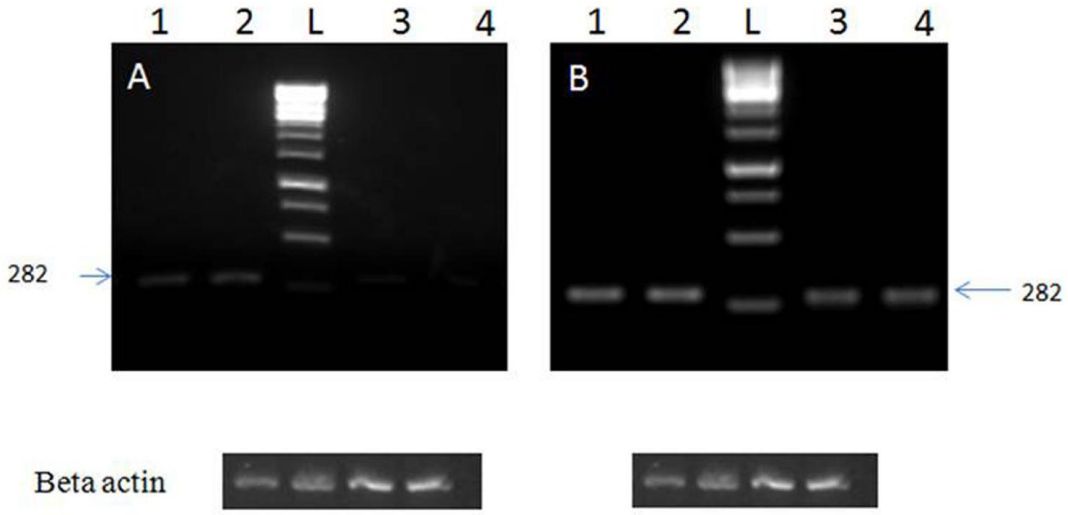

Figure 4: Electrophoretogram of Acetylcholinesterase (AChE) (A) Lane 1,2 Female cerebrum; Lane 3,4 Male cerebrum (B) Lane 1,2 Female Cerebellum; Lane 3,4 Male Cerebellum

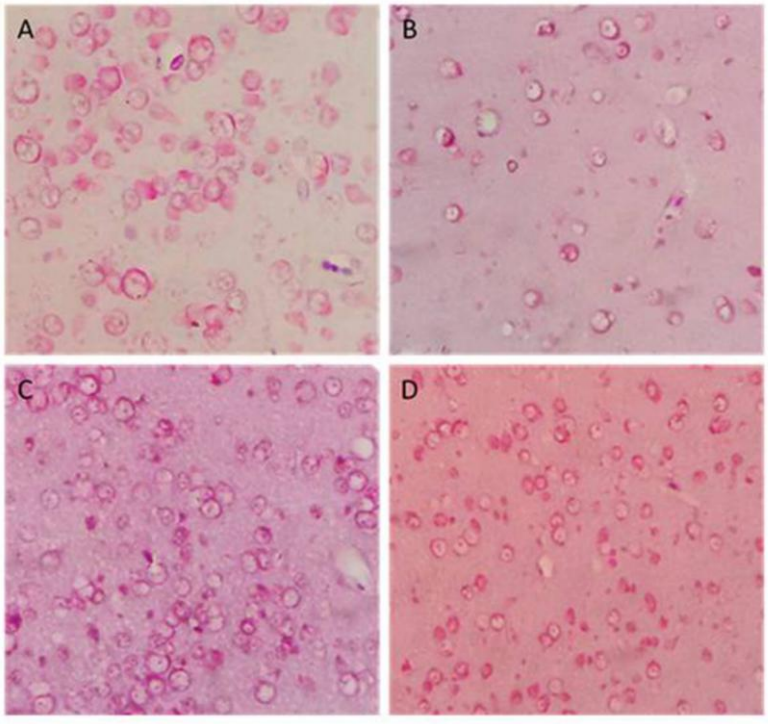

Figure 5: Methyl green-pyronin staining on *Duttaphrynus melanostictus* brain regions (A) Female Cerebrum showing maximum numbers of RNA granule (in comparison with Male cerebrum (B) Cerebellum, whereas again RNA granules are more in female cerebellum (C) with male cerebellum

Discussion:

AChEs have been so far identified in different tissues of most vertebrates and more than 20 invertebrate animals (10- 12). For instance, AChE activity has been detected in erythroid cells , brain, muscle, liver, kidney and lungs of vertebrates (13- 17), And it was also detectable in different tissues of invertebrates (18 – 22) , such as in the gills, mantle and haemolymph of mollusc (18, 19, 23), the eye and brain of arthropod (23), and the head of nematode (18, 22). There is a great difference in the amino acid sequence of AChEs from different animal, and it even varies greatly among the different tissues of the same organism (21). All the AChEs share some conserved structural features responsible for their catalysis function. For example, an active site triad (Ser, Glu and His) exist in all the reported AChEs, and the three residues form a plannar array at the bottom of a deep and narrow gorge, which closely resembles the catalytic triad of other a/b hydrolase fold family proteins (24).

Acetylcholinesterase (AChE; EC 3.1.1.7) in vertebrates was involved in cell development and maturation (25), neuronal development and nerve regeneration (26) and inflammation modulation (27). AChE had also been identified in most invertebrates, including mollusc, arthropoda, platyhelminthes, annelida and nematoda (28 – 32). And AChE was also reported to be involved in many behaviors in these invertebrates, including locomotion, feeding, egg laying, male mating, embryo development and digestive activity. However, the immunomodulation of AChE is still unclear in invertebrates. In the present study, an AChE gene was studied in male and female *Duttaphrynus melanostictus* cerebrum and cerebellum. The deduced protein of AChE (HM998937.1) was composed of 73 amino acids, and it shared 4.1% identity with other AChEs of cerebellum of male and female and there is no similar amino acids in cerebrum of male and female.

Inhibition of acetylcholinesterase (AChE)-metabolizing enzyme of acetylcholine, is presently the most important therapeutic target for development of cognitive enhancers. However, AChE activity in brain has not been properly evaluated on the basis of sex. In the present study, AChE activity was investigated in different brain areas cerebrum and cerebellum in male and female *Duttaphrynus melanostictus*. Females had a significant increase in AChE activity in cerebrum and cerebellum in comparision with male cerebrum and cerebellum. We also found that RNA granules are more in female cerebrum and cerebellum it may be one of the reason that affects the expression of AChE gene.

It is not possible, at present, to assign a definite factor to explain the pattern of deficit observed in the enzyme activity in male. Among the possibilities, decreased blood flow in brain causing hypoxia has been suggested for decrement in AChE turnover in whole brain of male (33, 34). Lack of uniformity in profile of AChE activity may be a reflection of functional heterogeneity in central cholinergic system observed by several workers on various parameters . In conclusion, the present study has provided an profile of AChE activity in major brain areas cerebrum and cerebellum of *Duttaphrynus melanostictus*. The activity in cerebrum and cerebellum indicates a possible pattern of distribution of G4 and G1 molecular forms of AChE in brain areas, respectively. It seems that the G4 form is dominant and is affected by sex as compared to G1. The variable enzyme activity in different brain areas and sex related

changes observed in male and female *Duttaphrynus melanostictus*, might be having an important bearing for functional heterogeneity reported for central cholinergic neuronal system and development of specific pharmacotherapy for cognitive disorders. Moreover, the study of the expression of AChE splice variants, their role in the neurotoxicity of organophosphate, contributes to the development of AChE gene expression as a new biomarker of susceptibility to improve the understanding of environmental and occupational health.

Conclusions:

Variable enzyme activity in different brain areas and sex related changes was observed in *Duttaphrynus melanostictus*, might be having an important bearing for functional heterogeneity reported for central cholinergic neuronal system and development of specific pharmacotherapy for cognitive disorders.

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