Award – winning oral presentation at SRBCE XXVIII, Jawaharlal Nehru University, New Delhi, February 4-6, 2010

Anti-/pro-oxidants stimulate thyroid hormone effects on amphibian metamorphosis: modulation through neurotransmitter turnover and reactive oxygen status in a tropical frog, *Clinotarsus curtipes* (Jerdon)

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Summary

Reactive oxygen species (ROS) are known to influence molecular and biochemical processes and signal transduction pathways, affecting cellular proliferation, differentiation and death in a variety of organisms. Amphibian metamorphosis encompasses all these three events within a short span of time. In the frog *Clinotarsus curtipes* development is largely akin to the post-embryonic development in mammals, displaying increased levels of ROS under *in vivo* physiological conditions. Scavenging ROS with an antioxidant revealed a serendipitous finding of turning to a pro-oxidant and a novel thyroid hormone mimetic with potential effects on neurotransmitter functions. Further, confirmatory studies, both conventional binding assays combined with *in- silico* approaches, revealed the ability of the compound to bind to human thyroid receptors thereby to mimic the thyroid hormone activity and thus function as potent endocrine disrupting chemical. Thus our study also cautions against the indiscriminate use of supplementary molecules without proper validation. Our studies on amphibian (*Clinotarsus curtipes*) development are valuable in examining the role of ROS in post embryonic development.

Key words : Neurotransmitters, metamorphosis, ROS, Oxidant**.**

Introduction

Amphibian metamorphosis is a complex morphogenic event that is obligatorily dependent on TH, with peaks of plasma TH levels coinciding with metamorphic climax (Brown, 2005; Valamparambil and Oommen, 1997). During metamorphosis there is *de novo* organogenesis and tissue remodeling, each involving a co-ordinated process of cell proliferation, differentiation, resorption and death (Denver, 1998). Amphibian larvae have tremendous capacity in behavior, morphology, growth and development rate which create the potential for the variation in the timing of, and size at, metamorphosis. Further, hormones of the neuroendocrine stress axis are reported to play crucial roles in mediating environmental effects on animal development (Denver, 2009). Anurans have formed the focal organisms in the study on amphibian metamorphosis, primarily due to the dramatic nature of metamorphosis and the ease in use of them in research. The tadpoles of *Clinotarsus curtipes*, the largest of any Indian tadpole known so far, are used as the model in the present study.

Neurotransmitters in the immature nervous system can act as trophic factors that influence different developmental events such as cell proliferation and cell differentiation. Antioxidant and anti-inflammatory therapy approaches have been in the focus of attention in the treatment of neurodegenerative diseases where oxidative stress has been implicated. Therefore, we employed a commonly used antioxidant propyl gallate (PG) to assess its potency *in vivo* in modulating tadpole metamorphosis, a thyroid hormone-dependent process and a focal model with similarities to postembryonic development in mammals. Both during normal development and after the administration of PG, the brain was assayed for the neurotransmitters (NT) catecholamines, viz., dopamine (DA) and homovanillic acid (HVA, a dopamine metabolite), and indoleamines viz., serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA, a 5-HT metabolite), reactive oxygen species, thyroid hormone receptor (B*max*, Kd), the biologically active thyroid hormone- L-triiodothyronine (T_3) levels, the endogenous tissue antioxidant enzymes- superoxide dismutase (SOD), and the lipid peroxidation index, malondialdehyde (MDA).

Materials and Methods

Clinotarsus curtipes tadpoles of various stages were maintained in the laboratory in fiberglass aquaria with aerated, de-chlorinated tap water at conditions of ambient temperature (29 ± 2°C) and photoperiod (approximately 12L:12D) and fed on boiled spinach (*Amaranthus* sp.)

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ad libitum. The tadpoles were staged on the basis of external morphology (Taylor and Kollros, 1946) (T-K) and morphometric index (Valamparampil, 1994).

Table 1

STAGES	HIAA/ HT	HVA/ DA
XVIII	158.92 ± 25.1 [a]	0.87 ± 0.12 [a]
XXI	103.22 ± 1.36 [b]	1.22 ± 0.13 [b]
XXIII	20.07 ± 3.0 [c]	1.27 ± 0.05 [b]
XXI + PG	243.42 ± 42.83*	2.33 ± 0. 63
XXIII + PG	45.02 ± 6.87 *	1 .25 ± 0.16

Table 1: Turnover of HIAA/HT and HVA/DA in control and PG-treated tadpoles of *C. curtipes* at different stages of metamorphosis. Groups with different letter headings are significantly different ($p<0.05$), as determined by one way ANOVA, using Duncan's multiple range test in SPSS. Values are means ± SEM of three separate experiments (n = 6 tadpoles per group). Significant difference between normal and treated groups were analyzed by Student's *t* test (*P< 0.05, when compared to control.

Twelve tadpoles each of pro-metamorphic stage XVIII were kept in 2 litres of water in two aquaria tanks. The normal tadpoles were in tank A and served as the control and those in tank B were the ones experimented upon. Propyl gallate (100 µM concentration based on our dose-determination study) dissolved in water was added to tank B and vehicle to tank A. The tadpoles in both the tanks were allowed to metamorphose. Both A and B groups of tadpoles changed from XVIII to climax stage XXI in about 2 days. Changes in external morphology were used as end point indices of assays. Therefore, there was no sampling of stage XVIII with PG treatment. Six tadpoles from tank A (normal) and six from tank B (PG-treated) were removed at stage XXI for analysis. The other tadpoles (6) in tank B reached stage XXIII in about three days and were sacrificed for analysis. However, the normal tadpoles in tank A took about 7 days to complete the metamorphosis. They were sacrificed thereafter. The water in both tanks was changed on alternate days and PG was added to tank B.

At the above mentioned time points, the frogs were removed from the tanks and their brains were dissected and kept at –80°C. The experiment was repeated in parallel three times to reconfirm the PG-induced metamorphosis and to have 6 tadpoles for each group of assays: 6 tadpoles in each group were used for enzyme assays, T_3 receptor and hormone assays, NT assays and 3-4 for histological and Western blot analysis. All assays were done according to standard protocols. At the end of each experiment (PG treatment)/developmental period, i.e., at stage XVIII, XXI and XXIII, the tadpoles were decapitated and the entire brain was used for analysis (the brain was too small to divide exactly into individual regions). The tadpoles were sacrificed between 8.00 and 10.00 am to avoid any circadian influence on the neurotransmitters studied. The care and treatment of animals used in this study were in accordance with institutional and national guidelines [G.O. (Rt) No. 240/07/F & Wld; IAEC-KU-2/05-06]. All reported values are mean ± SEM.

Table 2

STAGES	Bmax (fmoles/mg protein)	Kd (nM)
XVIII N	15.96 ± 0.24 [a]	0.516 ± 0.04 [a]
XXI N	20.54 ± 0.61 [b]	0.506 ± 0.03 [a]
XXIII N	18.07 ± 0.50 [c]	0.380 ± 0.02 [b]
XXI PG	121.0 ± 1.73**	1.16 ± 0.16*
XXIII PG	106.07 ± 1.51**	1.38 ± 0.09**

Table 2. T_3 B*max* and Kd in control and PG-treated tadpoles of *C. curtipes* at different stages of metamorphosis. Groups with different letter headings are significantly different ($p<0.05$) as determined by one way ANOVA, using Duncan's multiple range test in SPSS. Values are means ± SEM of three separate experiments (n = 6 tadpoles per group). Significant difference between normal and treated groups were analyzed by Student's *t* test (*P < 0.05, ** P < 0.01, when compared to control).

The data were statistically analyzed by one way analysis of variance (ANOVA). The significant difference among means was determined by Duncan's multiple range test (Duncan, 1955) at the level, $p<0.05$. Significant effects between normal and experimental groups were tested by means of paired sample Student's test.

Results and Discussion

The NT turnover and T_3 B*max* were examined in normal and PG-treated tadpole brain during different metamorphic stages. The turnover of HIAA/HT increased after PG treatment in metamorphic climax stages of XXI and XXIII (Table 1). Likewise, the HVA/DA ratio, which is also an indicator of oxidative stress, was increased by PG (Table 1). The T_3 concentration (Fig. 1A), B*max* (Table 2), TRα and TRβ (Fig.1B, C) expressions in the tadpole brain increased after PG treatment, resulting in advancement of metamorphosis. PG addition also

produced higher ROS levels as revealed by increased fluorescence of DHR and DCDFA probes, SOD protein and lipid peroxidation measured by MDA (Fig. 2A, B). Light microscopic observation of the control (Fig. 3A) and PG- treated brain (Fig. 3B) sections of stage XXIII tadpoles revealed numerous brown deposits of neuromelanin, which represents the end product of dopamine and norpinephrine oxidation pathways. In excess it becomes neurotoxic, largely by physical disruption of cellular function by massive amounts of the dense polymer (Smythies, 1999).

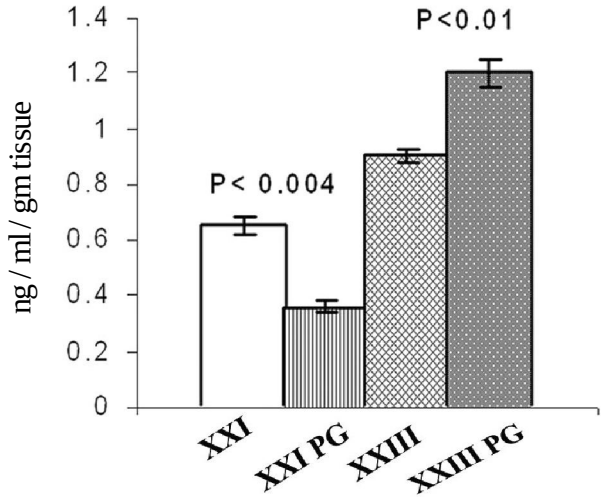

Fig. 1A: T_3 concentration in control and PG-treated tadpoles of *C. curtipes* at stages XXI and XXIII. Each histogram represents mean ± SEM of three separate experiments (n = 6 tadpoles per group). Significant difference between normal and treated groups were analyzed by Student's *t* test (P<0.01, P < 0.004, when compared to normal).

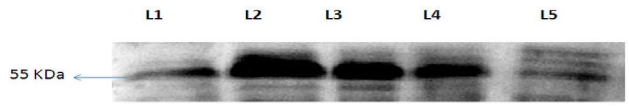

Fig. 1B: Western blot of tail TRα in control and PG-treated tadpoles of *C. curtipes*. Each lane (L1- L5) in the representative blot is of individual tadpoles from the normal metamorphosing and PG-treated stages. [L1 represents stage XVIII; L2- stage XXI normal; L3- stage XXI PG, L4- stage XXIII normal, L5- stage XXIII PG]*.

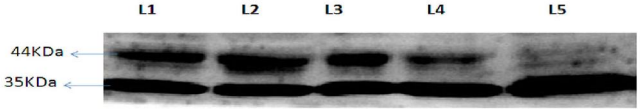

Fig. 1C: Western blot of tail TRβ in control and PG-treated tadpoles of *C. curtipes*. Each lane (L1-L5) in the representative blot is of individual tadpoles from the control and PG- treated stages.

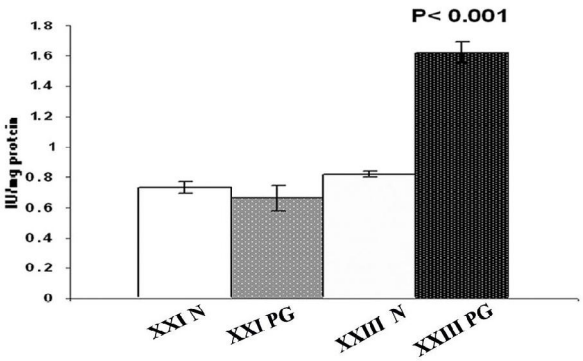

Fig. 2A: Relative analysis of the SOD native in-gel assay. The asterisk indicates a statistically significant (P>0.05) increase in the total level of SOD protein in PG-treated groups. In-gel Assay of SOD in control and PG-treated tadpoles of stages XVIII, XXI and XXIII. Each lane in the representative gel is of individual tadpoles from the normal and treated stages. The relative SOD level was measured by quantification of the intensity of the white band.

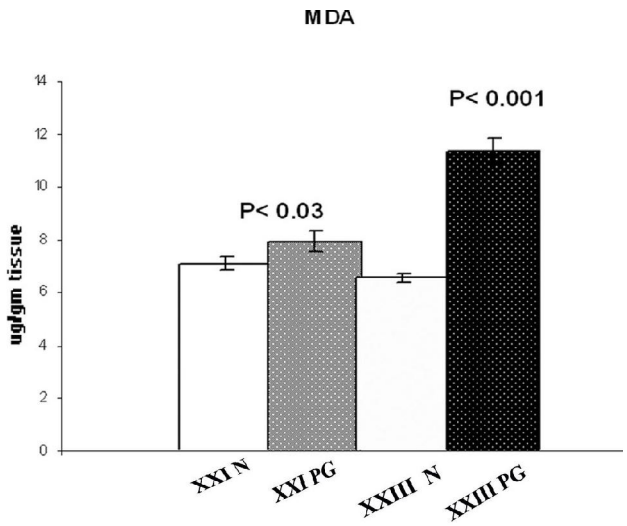

Fig. 2 B: Lipid peroxidation product, MDA, in control and PG-treated tadpoles of stages XXI and XXIII. Each histogram represents mean ± SEM of three separate experiments (n= 6 tadpoles per group). Significant differences between normal and treated groups were analyzed by Student's *t* test (P<0.03, P<0.001, when compared to control).

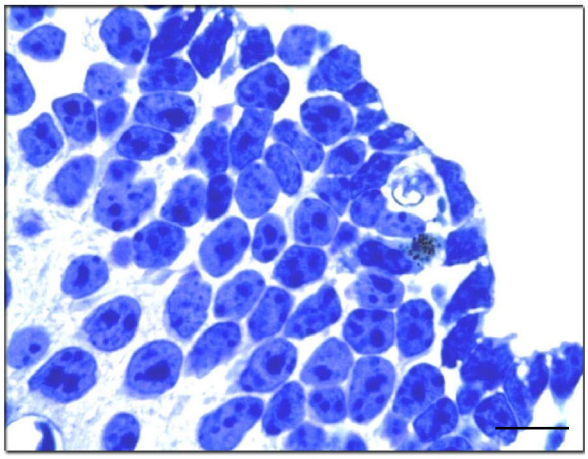

Fig. 3A: Light micrograph of brain semi-thin sections of *C. curtipes* at stage XXIII undergoing normal metamorphosis with sparse melanin.Scale bar = 20µ m.

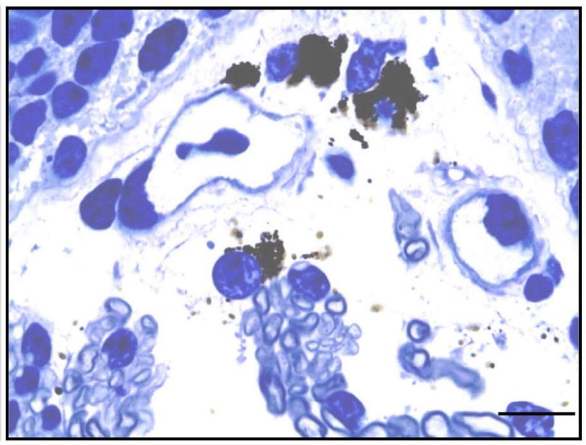

Fig. 3B: PG-treated stage XXIII tadpole brain, showing intense melanin pigmentation. Dark brown spots depict neuromelanin aggregation. Scale bar = 20 µm.

The normal balance of NT concentration during developmental stages of tadpole brain was modified by the pro-oxidant property of PG to result in early transformation of the tadpoles. We initiated the docking study to understand why PG functioned like the natural hormone, T_3, rather than inhibiting the action of the receptor. The molecular docking experiment is predicted with gold score and chemscore values which account for factors like ligand binding positions, H-bonding energy, van-der Waals energy and ligand torsion strain. The scores of more than 50% are considered significant (Table 3).

Table 3: Results of the molecular docking PG and T_3, showing Gold and Chemscore values.

Sl.No:	Protein	Ligand	Gold score	Chem. Score	Energy
1.	T3 RECEPTOR	T3	63	15	-2500.406
2.	T3 RECEPTOR	P G	72	19	-1083.476

Our wet lab experiments, comprising of competitive binding assays and Western blot analyses, confirmed the enhanced *in silco* binding of PG to TRs. Therefore, it is suggested that PG binds more effectively to thyroid hormone receptor compared to already existing T_3 molecule. Placing these observations in a structural context might aid discovery of selective receptor drugs such as antagonists or agonists, compounds that would have clinical value for the treatment of cardiac diseases and hypothyroid-associated ailments such as obesity and hypercholesterolemia. Therefore, our study further cautions against the indiscriminate use of antioxidant supplements, as the fine balance between oxidative stress and antioxidant status are delicately tuned.

Acknowledgments

The instrumentation facility under UGC, New Delhi, Grant No F 3-15/2007 SAP to Department of Zoology, University of Kerala, Thiruvananthapuram, is acknowledged. The first author (LD) acknowledges UGC Grant No F.4-3/2006 (BSR) RFSMS.

References

Brown DD (2005) The role of deiodinases in amphibian metamorphosis. *Thyroid* **15**:815-821.

Denver JR (1998) The molecular basis of thyroid hormone-dependent central nervous system remodeling during amphibian metamorphosis. *Comp Biochem Physiol. C* **119**:219-228.

Denver JR (2009) Stress hormones mediate environment-genotype interactions during amphibian development. *Gen Comp Endocrinol.* **164**:20-31.

Duncan DB (1955) Multiple range and multiple F test. *Biometrics* **11**:1-42.

Smythies J (1999) The neurotoxicity of glutamate, dopamine, iron and reactive oxygen species: functional interrelationships in health and disease: a review – discussion. *Neurotox Res* **24:**127-139.

Taylor AC, Kollros JJ (1946) Stages in the normal development of *Rana pipiens* larvae. *Anat Rec* **93**: 7-23.

Valamparampil TT (1994) *Endocrinological and morphological studies on the development and metamorphosis of Rana curtipes Jerdon*. Ph.D. Thesis. Thiruvananthapuram: University of Kerala.

Valamparampil TT, Oommen OV (1997) Triiodothyronine (T_3) and thyroxine (T_4) levels in *Rana curtipes* during development and metamorphosis. *Indian J Exp Biol.* **35**:1375- 1377.