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Effect of Physostigmine (an Organo Phosphate) on Biochemical variations of parotoid gland secretion and its extract of *Bufo melanostictus* (Schneider)

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Abstract

The present study was carried out to investigate the biochemical variations of parotoid gland secretion and its extract in common Indian Toad *Bufo melanostictus*. The parotoid glands were injected with an organophosphate compound, the physostigmine and the quantitative variations were observed in proteins, carbohydrates and ninhydrine positive substances at different time intervals i.e., 4, 8 and 12hrs. The results revealed that the components of proteins, carbohydrates and ninhydrine positive substances were found to be decreased significantly at 4, 8 and 12hrs time interval of Physostigmine in parotoid gland secretion compared to gland extract. The gradual decrease observed in biochemical constituents of both parotoid gland secretion and gland extract reveals triplicate response denoting the detoxification nature of OP compound i.e., Physostigmine. The maximum decrease in proteins followed by ninhydrin positive substances (free amino acids) and carbohydrates was observed at 4hrs and 12hrs compared to 8hrs time interval and control in both parotoid gland secretion and its extract.

Keywords: *Bufo melanostictus*, Carbohydrates, Ninhydrine positive substances, Physostigmine, Proteins.

1. Introduction

The presence of cutaneous granular glands is a shared character of amphibians and they are considered to be the source of bioactive compounds in amphibians (Daly *et al*., 1987). The glands in amphibian skin are fully developed. They are derived from epidermis and open outside through narrow ducts. There are two types of glands, the mucus glands, which are smaller in size and scattered throughout the skin, and poisonous glands, which are less abundant in their distribution and are often concentrated into specialized glands like parotoid glands in *Bufo* (Jared 2009). The secretions of mucus glands keep the skin moist and slippery, which enables them to escape from their predators

(Pedro *et al*., 2013); these glands secrete glycoprotein rich material which plays an important role in defense mechanism, while the parotoid glands located on dorsal side of the head, which ejects venomous secretion, known to be rich in biogenic amines, steroids, peptides and toxic defense molecules having medicinal applications against Cardiovascular disorders, Cancer and Diabetes (Parker and Hasswell, 1962; Clark 1997; Gomes *et al*., 2007a).

Organophosphate pesticides have gained popularity worldwide in preference to organochlorines, which are persistent and more damaging to the environment (Jaga and Dharmani 2003). Most of the countries have diverse amphibian population but it is surprising that much attention has not been paid to the effects of environmental pollutants upon these animals. The largest single group of potential chemical pollutants that frogs and toads might encounter is various pesticides employed in agriculture and pest management (Kumari and Sinha, 2006a). Pesticide effects on terrestrial life stages of amphibians are so far not accounted for amphibian conservation strategies where currently disease is discussed as a key factor (Fisher *et al*., 2012).

So far, there are few reports on the effect of organophosphate compounds (OP compounds) on biochemical variations in parotoid gland secretion and its extract. The present investigation has been undertaken to study the effect of Physostigmine (OP compound) on biochemical variations in parotoid gland secretion and its extract of *B. melanostictus*, in order to understand their possible role in potentiating/ detoxifying the venomous secretion in toads.

2. Experimental

The toads (7cm to 10 cm in length, weighed about 50 to 75 grams.) were collected from vicinity of Kakatiya university hostel buildings, Warangal, A.P, India. The parotoid glands were gently pressed to release the secretion with the help of sterile forceps. The secretions were collected into ice-jacketed containers. After collecting secretions, the gland was dissected out, blotted to free from blood clots and other adherent tissues and weighed to the nearest milligram and processed for further analysis. They were homogenized (10%) in 10% Tri Chloro Acetic Acid (TCA) to sediment of protein. The protein sediment was dissolved in 1N NaOH and protein content was determined through the Lowry's reagent (1951) described by Schacterle and Pollack (1973). The TCA supernatant was used to estimate TCA soluble peptides (Lowry's reagent), Ninhydrine positive substances (Lee and Takahashi, 1966) and Carbohydrates (Anthrone method, Carroll *et al*., 1956).

For the estimation of biochemical variations in *B. melanostictus* an organophosphate (OP) compound, Physostigmine (10^{-4}M) and normal saline, were injected intradermally into parotoid gland contra laterally. The *in vivo* effects of Physostigmine in biochemical variations of parotoid gland secretion and its extract of *B. melanostictus* were studied according to the procedures of Lowry, Schacterle and Pollack, Lee and Takahashi and Carroll at different time intervals i.e., 4, 8 and 12hrs.

Data Analysis:

In this experiment, when parotoid glands of toad were injected with the desired concentrations of the test chemical Physostigmine at different time intervals, a drastic reduction was observed in total biochemical constituents in parotoid gland secretion and its extract. One way ANOVA (Dunnett's test) was used to compare the results between the tissue components.

3. Results and Discussion

The values obtained from the quantitative estimates on effect of Physostigmine on biochemical variation, in parotoid gland secretion and its extract of *B. melanostictus* are presented in the Tables-1, 2, 3 and Figure 1, 2, 3 respectively. After injection of physostigmine into parotoid gland, the maximum reduction in biochemical variations was observed in both parotoid gland secretion and its extract compared to control.

In parotoid gland secretion, maximum reduction was observed in TCA soluble proteins and TCA precipitated proteins at 4 hrs time interval and were noted as 35.83 mg/gram (-56%) and 24 mg/gram (-70%) respectively. In case of parotoid gland extract, the TCA soluble proteins and TCA precipitated proteins at 4 hrs time interval were found to be 33.25 mg/gram (-40%) and 28.5 mg/gram (-57%) respectively.

At 8 hrs time interval, TCA soluble proteins and TCA precipitated proteins were found to be 27 mg/gram (-67%) and 9.5 mg/gram (-88%) respectively in parotoid gland secretion, while in parotoid gland extract, the TCA soluble proteins and TCA precipitated proteins were noted as 18.5 mg/gram (-66%) and 10.75 mg/gram (-83%) respectively. At 12 hrs time interval, TCA soluble proteins and TCA precipitated proteins of parotoid gland secretion were found to be 14.6 mg/gram (-82%) and 7.41 mg/gram (-90%) respectively. While in parotoid gland extract, TCA soluble proteins and TCA precipitated proteins were observed as 7.75 mg/gram (-86%) and 7.08 mg/gram (-89%) respectively.

Table 1: Biochemical variations of proteins after inducing the Physostigmine in the Parotoid gland secretion and its extract of *Bufo melanostictus*

Period of Exposure	Parameter	Variation of protein content after induction of Paraoxon			
		Parotoid gland Secretion	% of Change	Parotoid gland extract	% of Change
Control	TCA soluble proteins	82.75 ± 0.58	21%	55.83 ± 0.89	11%
Control	TCA precipitated proteins	80.35 ± 0.62	47%	67 ± 0.81	16%
4 hrs	TCA soluble proteins	35.83 ± 0.16***	-56%	33.25 ± 0.10***	-40%
4 hrs	TCA precipitated proteins	24 ± 0.11***	-70%	28.5 ± 0.45***	-57%
8 hrs	TCA soluble proteins	27 ± 0.46***	-67%	18.5 ± 0.48***	-66%
8 hrs	TCA precipitated proteins	9.5 ± 0.11***	-88%	10.75 ± 0.16***	-83%
12 hrs	TCA soluble proteins	14.6 ± 0.30***	-82%	7.75 ± 0.52***	-86%
12 hrs	TCA precipitated proteins	7.41 ± 0 .75***	-90%	7.08 ± 0.41***	-89%

Values are expressed as fresh weight of tissue mg/gram mean ± SE; n = 6,
Statistically significant value to respective control value * P<0. 05, ** P<0.01, *** P<0.001.

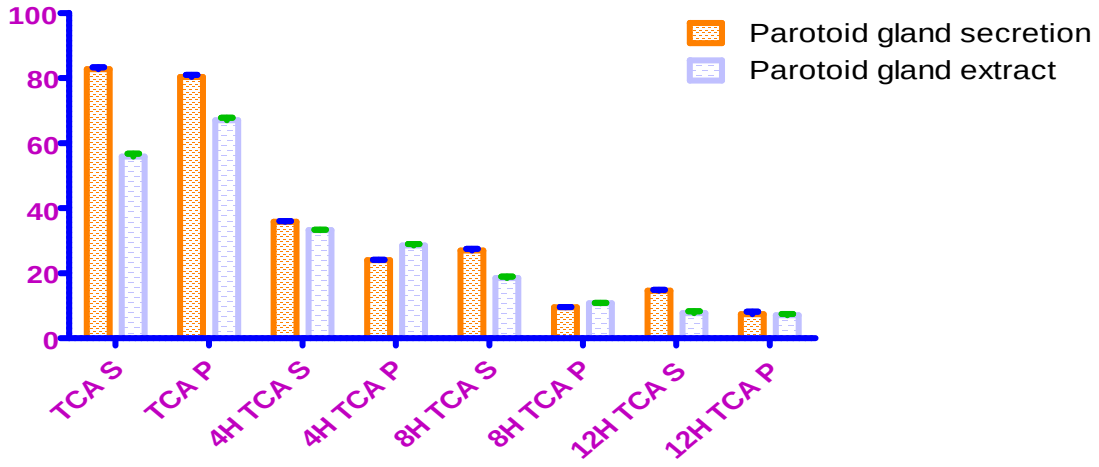

Figure 1: Biochemical variations of proteins after inducing of physostigmine in the Parotoid gland secretion and its extract of *Bufo melanostictus*

TCA S-TCA soluble proteins,
TCA P- TCA precipitated proteins.

The results presented in Table-1 and Figure-1 shows that the protein content was decreased in supernatant treated with Physostigmine. After 4 and 8 hrs induction, the reduction in soluble protein content (TCA soluble proteins) and the structural protein content (TCA precipitated proteins) was found to be $p < 0.001$ in parotoid gland secretion and its extract.

At 4 hrs time interval, the reduction in the carbohydrate content was 2.16 mg/gram (-47%) and 1.08 mg/gram (-76%) in the parotoid gland secretion and its extract, while at 8 hrs time interval the reduction was 2.25 mg/gram (-44%) in the parotoid gland secretion and 2.25 mg/gram (-51%) in parotoid gland extract whereas at 12 hrs time interval the reduction was 2.33 mg/gram (-42%) and 2.75 mg/gram (-40%)in the parotoid gland secretion and parotoid gland extract respectively.

Table 2: Biochemical variations of Carbohydrates after inducing Physostigmine in the Parotoid gland secretion and its extract of *Bufo melanostictus*

Period of Exposure	Variation of carbohydrate values after inducing of Physostigmine			
	Parotoid gland secretion	**% of Change**	**Parotoid gland extract**	**% of Change**
Control	4.08±0.08	1%	4.66±0.10	0.5%
4Hours	2.16±0.10***	-47%	1.08±0.11***	-76%
8Hours	2.25±0.25***	-44%	2.25±0.08***	-51%
12Hours	2.33±0.10***	-42%	2.75±0.11***	-40%

Values are expressed as fresh weight of tissue mg/gram mean ± SE; n = 6,
statistically significant value to respective control value
* P<0. 05,
**P<0.01,
***P<0.001.

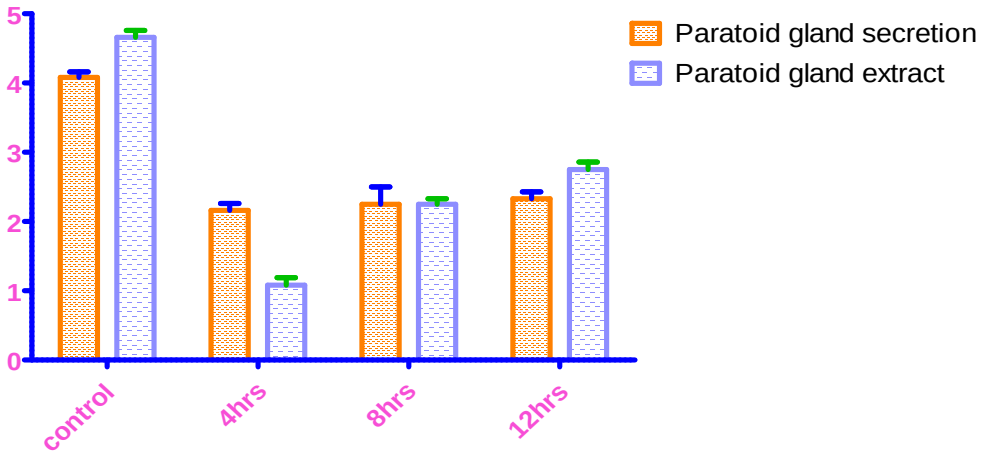

Figure 2: Biochemical variations of Carbohydrates after inducing of Physostigmine in the Parotoid gland secretion and its extract of *Bufo melanostictus*.

The results presented in Table-2 and Figure-2 revealed that the carbohydrate content was decreased in parotoid gland secretion and its extract when compared to control. In our observations, Physostigmine treated samples with 4, 8 and 12 hrs treatment, the p value of carbohydrate content was found to be significant with p <0.001 in parotoid gland secretion and its extract. Hence, it can be concluded that there is a significant variation between the parotoid gland secretion and its extract.

Table 3: Biochemical variations of Ninhydrine positive substances (Free aminoacids) after inducing of Physostigmine in the Parotoid gland secretion and its extract of *Bufo melanostictus*

Period of Exposure	Variation of Free amino acid values after inducing of Physostigmine			
	Parotoid gland secretion	**% of Change**	Parotoid gland extract	**% of Change**
Control	5.08 ± 0.83	18%	18.3 ± 0.62	0.2%
4H Dose	2.10 ± 0.54**	-58%	2.5 ± 0.32***	-86%
8H Dose	3.33 ± 0.25^{ns}	-34%	5 ± 0.54***	-72%
12H Dose	4.16 ± 0.45^{ns}	-18%	1.66 ± 0.11***	-90%

Values are expressed as fresh weight of tissue mg/gram mean ± SE; n = 6,
Statistically significant value to respective control value
*P<0. 05,
** P<0.01,
*** P<0.001.
ns- Non significant

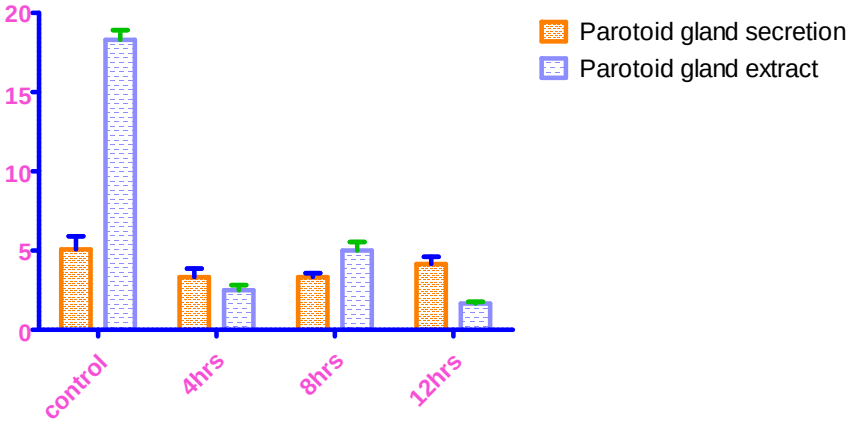

Figure 3: Biochemical variations of Ninhydrine positive substances after inducing the Physostigmine into the Parotoid gland secretion and its extract of *Bufo melanostictus*.

The results presented in Table-3 and Figure-3 revealed that the ninhydrine positive substances (free amino acids) were decreased in parotoid gland secretion and its extract compared to control with $p<0.05$ at 4 hrs, while non significant at 8 and 12hrs time intervals in gland secretion where as the gland extract also showed decreased free amino acid content with a significant value of $p<0.001$ at 8 and 12 hrs time interval.

At 4 hrs time interval, the ninhydrin positive substances in the parotoid gland secretion and its extract were observed as 2.10 mg/gram (-58%) and 2.5 mg/gram (-86%) respectively. While at 8 hrs time interval, the reduction was 3.33 mg/gram (-34%) in the parotoid gland secretion and 5 mg/gram (-72%) in parotoid gland extract and at 12 hrs time interval the reduction was 4.16 mg/gram (-18%) in the parotoid gland secretion and 1.66 mg/gram (-90%) in parotoid gland extract. The ninhydrin positive substances in the parotoid gland secretion found to be non significant statistically and showed significant reduction only in the parotoid gland extract i.e., 1.66 mg/gram (-90%). The maximum decrease was observed at 4hrs and 12hrs time intervals compared to 8hrs time interval and control, indicating the detoxification nature and re potentiating capacity of esterase which helps in the predation and self defense mechanism of toad parotoid glands.

The organophosphate compounds are widely used as insecticides and extremely toxic in some cases, these materials are generally short lived in the environment compared to halogenated organics and related compounds (Mahananda and Mohanty, 2012). Amphibian populations are declining globally at an alarming rate. Pesticides are among a number of proposed causes for these declines. Although a sizable database examining effects of pesticides on amphibian exists, the vast majority of these studies focus on toxicological effects (lethality, external malformations, etc.) at relatively high doses (parts per million). Very few studies focus on effects such as endocrine disruption at low concentrations (Tyrone *et al*., 2006). Further, most studies examine exposures to single chemicals only. Although a sizable database examining the toxicological effects of pesticides on amphibians exists (Pauli 2004), most of these studies examine acute toxicity, morbidity and mortality only.

Amphibians especially in agricultural areas are exposed to mixtures of pesticides. Adverse effects are due to the continuous use of pesticides in agriculture over the last 50 years, which have played and will continue to play a role in amphibian declines. In particular, the effects described here are very important. Pesticide-induced declines in populations as a result of decreased prey availability and increased susceptibility to predators may be difficult to discern in the wild (Tyrone *et al*., 2006).

Our present investigation reports on effect of physostigmine, an organophosphate compound on biochemical variations of parotoid gland secretion and its extract of *B. melanostictus* and revealed that the total protein content found to be decreased in both parotoid gland secretion and its extract in Indian Toad after injection of Physostigmine. The decrease of total protein content in both parotoid gland secretion and its extract may be due to less incorporation of amino acids in the translation process i.e., a reduced incorporation into any kind of protein and the disturbance caused by the pesticides during protein synthesis (Stuart *et al.*, 2004).Regarding pesticides, the present study demonstrates that the examinations of effects of single pesticide are inadequate to assess adverse impacts on amphibian development or to address the role of pesticides in amphibian declines. The examinations needed to characterize pesticide interactions as concentration additive or response additive (Burkhart *et al*., 2003); to

distinguish between effectors and enhancers, and to examine multiple combinations of pesticides at multiple concentrations are difficult to design.

4. Conclusion

The results from present studies showed that the parotoid gland secretion and its extract were rich in protein content compared to carbohydrate and free amino acids and were more sensitive to OP compound, effecting their physiological activities and xenobiotic metabolism which are species specific, this needs further investigation.

5. Acknowledgements

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