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Cardiotonic activity of Parotoid gland secretion of common Indian Toad Bufo melanostictus on isolated heart of Frog

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ABSTRACT:*The present investigation has been undertaken to study the cardiotonic activity of parotoid gland secretion of common Indian Toad Bufo melanostictus on isolated perfused frog heart (Rana tigrina). Cardiotonic activity of parotoid gland secretion of B. melanostictus in normal as well as when induced with paraoxon (an organophosphate) was studied. For the evaluation of cardiotonic activity, Syme's technique is being employed, digoxin and propranolol were used as standard drug and β-blocker respectively to characterize the effects on the receptors. The isolated perfused frog heart and hypodynamic frog heart showed dose dependent positive ionotrophic effects. The parotoid gland secretion exihibited cardiac stimulant activities. The propranolol was not able to block the effects of paraoxon induction on toad secretion. Thus, the present investigation reports that the parotoid gland secretion increased the force of contraction, heart beat and cardiac output in perfused frog's heart, whereas, there was no change on hypodynamic heart, indicating that there may be existence of two components, one with β–receptor stimulating activity and other acting directly on the heart (independent of β_1-adrenoreceptors).*

KEY WORDS*: Bufo melanostictus, Rana tigrina, Cardiotonic activity, Parotoid gland secretion, Paraoxon.*

I. INTRODUCTION

In recent years there has been an increased interest in search of new pharmaceutical compounds of natural origin has intensified and been extended to include sources other than plant material **[**1].The alarm/defense secretions of Amphibians also exhibit a number of pharmacological activities and quite a good number of pharmacologically active substances like pahutoxin [2], bufotenin and bufotoxins [3], biogenic amines [4], bioactive peptides [5,6 and 7] have been isolated in purified form from the skins of amphibians.

Amphibian skin is characterized by the presence of cutaneous glands spread over the body. Basically toads have two types of alveolar glands in the epidermal layer of their skin i.e. (i) mucous glands and (ii) granular glands [8, 9]. Mucous glands secrete mucus, functioning as a lubricant in the water to keep skin moist and necessary for cutaneous respiration and protect the skin from mechanical damages and prevent microbial settlement on the skin. Skin plays an important role in defense mechanism and mucus known to be glycoproteinaceous being rich in a variety of protein residues such as mucin, musinigen, and carbohydrate residues such as galactose, fukosa, and sialaic acid. Granular glands are also called poison glands which secrete serous that provide protection from predators such as birds, mammals, snakes and crocodiles [10].

The parotoid gland of the *Bufo* species has been known to secrete several bio active compounds like bufotenins, bufalins and bufotoxins [3] .The venomous secretions of these glands were also used as cardiotonic drugs by Chinese and Japanese physiscians in folk medicine. The toad venom especially bufotenin secreted by these glands was subject for mythological, pharmacological and medicinal practices for centuries [11]. The Scientific research has confirmed the presence of bufalin in "Chan Su" a traditional Chinese medicine prepared from dried white secretion of the skin glands of Chinese toad used as a drug for treating heart disorders, heamorrhage of gums, sinusitis and other systemic illnesses [12].

In view of the importance of compounds present in the skin secretions of parotoid glands to pharmacology, especially to heart physiology, an investigation was carried out on the effects of the parotoid gland secretion of toad *Bufo melanostictus*, on the isolated hearts of the frog *Rana tigrina*.

II. MATERIALS AND METHODS

2.1 Studies on Heart beat:

The toads (7cm to 10 cm in length, weighed about 50 to 70 gm.) were collected from the vicinity of Kakatiya University hostel buildings, Warangal, A.P., India. The parotoid glands were gently pressed with the help of sterile forceps to release the secretions according to Meyer and Linde (1971) [13] and these secretions were collected into ice-jacketed containers. The secretions were weighed to nearest milligram. They were stored in cool conditions until further analysis. The secretion and the glands were weighed to the nearest milligram and were homogenized (10%) in cold 0.9% of normal saline or frog Ringer's solution. The secretions were vortexed for 5 minutes and centrifuged at 2000 rpm for 20 minutes. The freshly collected compounds were dissolved in normal saline/frog ringer's solution to study the effect on isolated hearts of freshly killed *Rana tigrina.* The isolated heart mounted on to Syme's cannula [14] was used for the study.

2.2 Preparation of isolated hearts for experiment:

Frog (*Rana tigrina*) was stunned by head-blow using a steel rod and pithed. The skin and abdomen were cut and opened. The pectoral girdle was cut using a bone cutter and pericardium was cut and removed. Syme`s cannula was connected to the reservoir of frog Ringers solution and introduced immediately into the Sinus venosus of the heart. Frog Ringer's solution was immediately introduced into the sinus venosus of the heart through posterior venacava after pericardium and the connecting blood vessels were removed and the heart was removed from the animal and mounted on to the stand. The heart was covered with thin layer of cotton and was wetted continuously with frog Ringer's solution to prevent the drying of the tissue. The heart was connected to the starting lever, which was in turn connected to kymograph drum for recording the heart beat. The flow of Ringer's solution into Syme's cannula was maintained by fixing a glass tube into the cork fixed to the reservoir (Marriott bottle) tightly. The heart was allowed to stabilize and when the heart rate and cardiac output were stabilized, the recordings made on a slowly rotating drum to which sooted kymograph paper was affixed. The effects of parotid secretion *per se* (in Ringer) and its fraction were studied on isolated perfused frog hearts. The parameters studied included the force of contraction, heart rate and cardiac output. The volume of frog Ringer's solution coming out of the heart per minute (Cardiac output) was measured by collecting the solution into a measuring jar. The heart rate was measured by counting the number of heart beats per minute. Minimum 5 min time was allowed between the additions of parotoid exudates *per se* (in Ringer) and its fraction. When blocker was used, it was diluted with known amount of the physiological solution in syringe itself and added slowly.
The heart rate (HR), cardiac output (CO) and force of contraction were the parameters used for the study. The solutions of parotoid gland secretion per se and the extracted fractions were prepared in frog Ringer's solutions. No suspending agents were used. The heart was constantly moistened with frog's Ringer solutions from time to time.

2.3 Hypo Dynamic Frog's Heart

An isolated frog heart preparation as described under Syme's technique was set up. Instead of one reservoir, two reservoirs each for ½ calcium and full calcium were used. The levels in the reservoir maintained constantly, which was tested by connecting each of the reservoirs to the Syme's cannula.

Experiments were conducted by rendering the frog heart hypodynamic by letting into heart, frog ringer's containing ½ calcium from another reservoir through syme's cannula. Force of contraction was monitored to give half the magnitude of normal force of contraction. The effects of parotoid gland secretion on hypo dynamic hearts were determined by using frog Ringer with ½ calcium concentration. Propranolol, the non specific β-blocker was used to characterize the effects on the receptors.

Studies were conducted on 6 animals and each time collections from individual toads were used to see the reproducibility of the effects. Samples were given as spot dose into Syme's cannula through a tuberculin syringe. The heart rate and the cardiac output were measured simultaneously. Dose-dependent effects of the parotoid gland secretion were noted to get optimum results.

2.4 Preparation of digoxin solution:

The marketed digoxin gift samples (Sunpharma Ltd.) were obtained from local market. Various different dilutions were made with distilled water and labeled as follows, B1- 25 µg/ml**,** B2- 50 µg/ml. above prepared samples were evaluated for their cardiotonic activity and treated as standard.

2.4 Composition and Preparation of hypodynamic Ringer's solution

Hypodynamic ringer solution was prepared by using standard method [15].

Sr. No.	Ingredients	Quantity
1	Sodium chloride (NaCl)	6.5 gm
2	Potassium chloride (KCl)	0.14 gm
3	Calcium Chloride ($CaCl_2$)	0.03 gm
4	Sodium bicarbonate ($NaHCO_3$)	0.2 gm
5	Glucose	2.0 gm
6	Distilled Water	1000 ml

2.5 Preparation of OP Compound concentrations for induction to study Pharmacological studies [16].

To observe the pharmacological effects after exposure of OP compound, Paraoxon (0, 0-di-ethyl-4-nitrophynyl phosphate ($2X10^{-5}M$)), concentration in the parotoid gland secretion and its extract. The OP compound concentrations and normal saline were induced sub-cutaneously into parotoid gland contra laterally. The *in vivo* effects of Paraoxon, on cardiotonic activity of parotoid gland secretion and its extract were noticed after 4 hours time interval.

III. RESULTS AND DISCUSSION

The results on cardiotonic activity of paraoxon induced and normal parotoid gland secretions (without induction) are presented in Tables 1&2, Fig 1&2 and Graphs.1&2. The results indicated that the parotoid exudates on isolated perfused frog hearts elicited dose dependent effect. There was an increase in force of contraction (the positive ionotropic effect) *per se* (in ringer) and slight increase in cardiac output (Tables, Fig and Graphs.1&2). Propranolol was not able to block the response of parotoid exudates *per se* (in Ringer) totally (Figure. 1&2). A partial reduction in amplitude of contraction was noticed (Fig-1&2), indicating that there may be two components, one with β–receptor stimulating activity and other acting directly on the heart (independent of β_1-adrenoreceptors). The gland extracts exihibit the cardiac stimulant activities (Fig-1&2). The dose dependent changes were measured in heart rate (HR), cardiac output (CO) and percentage of increase in force of contraction (% IFC). In all these dose concentrations Parotoid gland exudates exhibited prominent cardio tonic effect in hypodynamic heart. Propranalol was not able to block the total amplitude of force of contraction but reduction in the force of contraction indicates dual roles like cardio tonic and cardiac stimulant activities.

On hypodynamic heart normal parotoid gland secretion showed dose dependent changes in heart rate (HR), cardiac output (CO) and percentage of increase in force of contraction (% IFC). The normal parotoid gland secretion showed dose dependent changes on isolated heart, and the results were observed as 42 beats/min (HR), 10 ml/min (CO). At 0.1 ml (50µg) dose, the heart rate was noted as 40 beats/min (HR), cardiac output of 12 ml/min (CO) and with an increase of 50% in force of contraction (% IFC). At 0.2 ml (100µg) dose, the results obtained were 38 beats/min (HR), 15 ml/min (CO) and 70% of increase in force of contraction (% IFC). At 0.3 ml (150µg) dose, results were 36 beats/min (HR), 16 ml/min (CO) and 90% of increase in force of contraction (% IFC), while at the standard digoxin dose (1000ng), the results were 38 beats/min (HR), 16 ml/min (CO) and 30% increase in force of contraction (% IFC). At the maximum concentration of propranolol and the maximum dose of parotoid gland secretion i.e., 0.3 ml (150µg), the results obtained were 38 beats/min (HR), 14 ml/min (CO) and 20% increase in force of contraction (% IFC) which revealed that the Propranolol was not able to block the response of parotoid exudates *per se* (in Ringer) totally.

The bufotenins, bufalins, bufotoxins of toads [3] and bufalins present as free genins or as conjugates of steroids in these secretions were reported to be similar to digitalis in their action. Further studies [17], suggested that the cardiotonic compounds affect the force of contraction without affecting the heart rate significantly. The results obtained in the present investigation are consistent with above observations. The parotoid gland extracts exhibit the cardiac stimulant activities [18, 19] and the OP compounds like Paraxon could not inhibit the cardiotonic activity of parotoid gland exudates. OP compounds inhibit the level of biochemical constituents and esterase activities [20].

3.1 The effect of Paraoxon

The effect of paraoxon (OP) induced parotoid gland secretion revealed that the cardiac output and increase in force of contraction (the positive inotropic effect) *per se* (in ringer) increased on isolated heart and hypodynamic heart (Table, Fig and Graph-2) compared to normal parotoid gland secretion (Table, Fig and Graph-1). The effect of Paraoxon induced parotoid gland could not decrease cardio tonic and cardiac stimulant activities of parotoid gland secretion. Propranolol was unable to block the amplitude of force of contraction in the Paraoxon induced parotoid gland secretion (Fig-2) but increase in activity compared to normal parotoid gland secretion (Figure-1) was noticed.

On hypodynamic heart paraoxon induced parotoid gland secretion showed dose dependent changes in normal and the results were observed as 55 beats/min (HR), 12 ml/min (CO), while paraoxon induced parotoid gland secretion showed dose dependent changes at 0.1 ml (50µg) with 53 beats/min (HR), 14 ml/min (CO) and 50% increase in force of contraction (% IFC) while at 0.2 ml (100µg) dose results were 50 beats/min (HR), 16 ml/min (CO) and 70% increase in force of contraction (% IFC). At the 0.3 ml (150µg) dose, the results were 50 beats/min (HR), 18 ml/min (CO) and 90% increase in force of contraction (% IFC). At the standard digoxin dose (1000ng) results were observed to be 48 beats/min (HR), 20 ml/min (CO) and 20% increase in force of contraction (% IFC). The Propranolol and paraoxon (the maximum dose) induced parotoid gland secretion, i.e., at 0.3 ml (150µg) results obtained were 54 beats/min (HR), 14 ml/min (CO) and 40% increase in force of contraction (% IFC) reveals that the Propranolol was not able to block the response of parotoid exudates *per se* (in Ringer) totally **(**Fig, Table & Graph-1&2).

Toad-toxins act by inducing various physiological effects on higher as well as lower vertebrates. Granular gland secretions when entered into stomachs of higher-vertebrates cause nausea, weakening of respiration and muscular paralysis, while in contact with eyes they produce serious inflammations [21]. Toad secretions may also boast of adrenalin, which is a result of chemical change within mature secretion [22]. Skin Clinical aspects of toad-toxins have been studied particularly in *Bufo marinus.* Secretions of *B. marinus* are cardio active, due to activity of bufogen and bufotalin exhibiting clinical symptoms like dermatitis, hypotension and severe arrhythmia [23]. Toad-secretions have also been found to show second-degree Wenckebach atrio ventricular block and T-wave change [24].

Bufogenines are the inhibitors of the enzyme Na^+/K^+ ATPase activity in the myocardial cell memebrane [25] acting evidently through the same mechanism. Bufonid toad's skin of the genera *Atelopus, Dendrophryniscus and Melanophryniscus* contain Bufadienolides or related compounds on basis of assays of inhibition of Na^+/K^+ ATPase or binding of titrated ouabin [25].

IV. CONCLUSION

It will be interesting to isolate the active chemical constituents which are responsible for the cardiotonic activity as well as to determine the possible mechanism of action. It is possible that the peptide or an amine or may be the other compound effecting the cardiac activity.

Table-1 Effect of parotoid gland secretion on isolated frog heart (the parameters include the force of contraction, heart rate and cardiac output.)

Dose	**Result**			
On Normal Heart	HR (beats/min)	CO (ml/min)	Increase in force of contraction (in mm)	% Increase in force of contraction
Normal	48	12.5	-	-
0.1 ml(50 µg)	50	13	5	50
0.2 ml(100 µg)	52	13	3	30
0.3 ml(150 µg)	54	13	2	20
Digoxin 400 ng	48	12	0.5	5
Digoxin 1000 ng	48	14	1	10
On Hypodynamic heart				
Normal	42	10	-	-
0.1 ml(50 µg)	40	12	5	50
0.2 ml(100 µg)	38	15	7	70
0.3 ml(150 µg)	36	16	9	90
Digoxin 1000 ng	38	16	3	30
PP+0.3ml(150 µg)	38	14	2	20
Normal	42	11	-	-

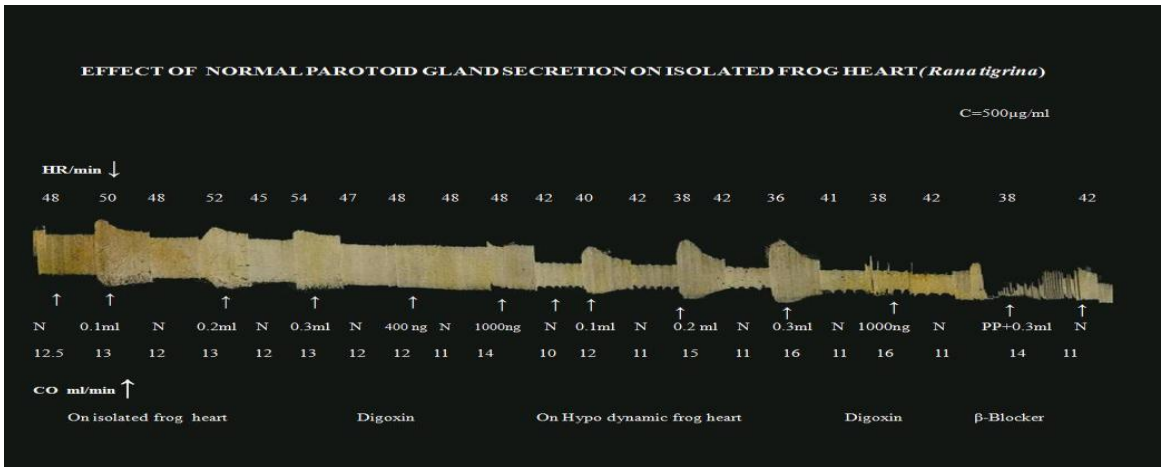
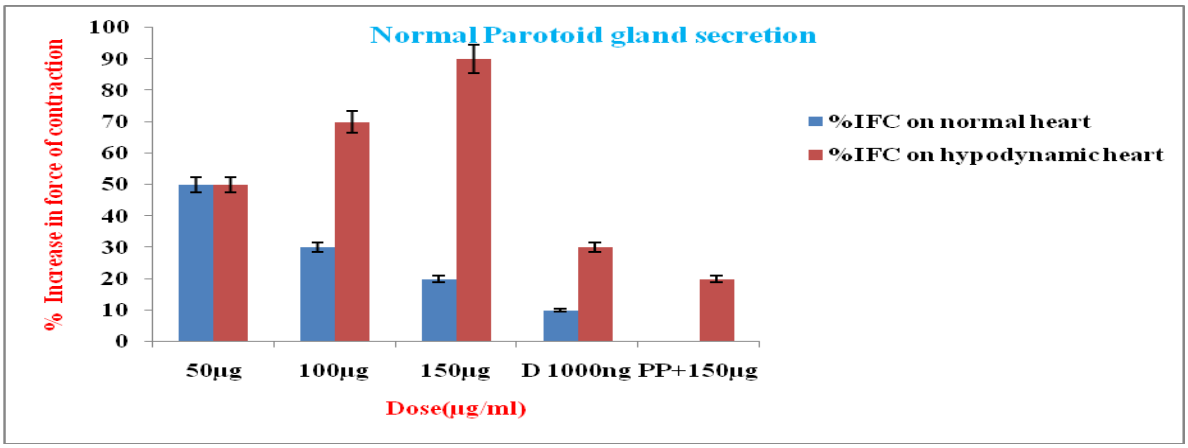


Fig-1 Effect of parotoid gland secretion on isolated frog heart the parameters includes the force of contraction, heart rate and cardiac output.



Graph-1 Effect of parotoid gland secretion on isolated frog heart elicited a dose dependent increase in force of contraction (positive inotropic effect).

Table-2 Effect of Paraoxon induced parotoid gland secretion on isolated frog heart the parameters includes the force of contraction, heart rate and cardiac output

Dose	**Result**			
On Normal Heart	HR (beats/min)	CO (ml/min)	Increase in force of contraction (in mm)	% Increase in force of contraction
Normal	60	12	-	-
0.1 ml(50 µg)	58	14	2	20
0.2 ml(100 µg)	56	16	4	40
0.3 ml(150 µg)	58	16	5	50
Digoxin 400 ng	60	12	1	10
Digoxin 1000 ng	56	14	1	10
On Hypodynamic heart				
Normal	55	11	-	-
0.1 ml(50 µg)	53	14	5	50
0.2 ml(100 µg)	50	16	7	70
0.3 ml(150 µg)	50	18	9	90
Digoxin 1000 ng	48	20	2	20
PP+0.3ml(150 µg)	54	14	4	40
Normal	58	12	-	-

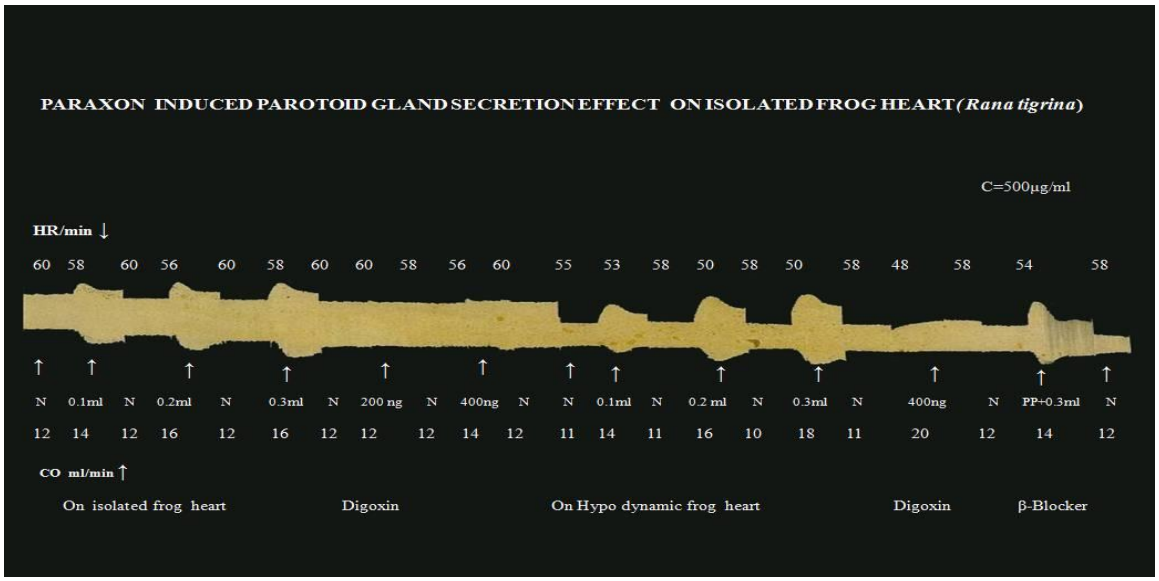
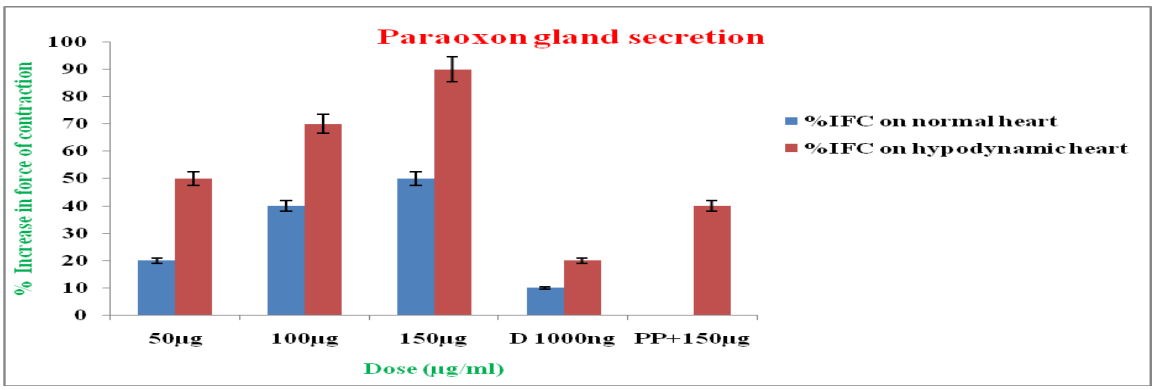


Fig-2 Effect of Paraoxon induced parotoid gland secretion on isolated frog heart the parameters includes the force of contraction, heart rate and cardiac output.



Graph-2.Effect of Paraxon induced parotoid gland secretion on isolated frog heart elicited a dose dependent increase in force of contraction (positive inotropic effect).

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