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The emblem of Pranikee

The emblem "***NABAGUNJARA***" is a chimeric animal and a common motif of Orissan art and literature. It literally means "Nine form". This form has been described by poet Sarala Das in the Oriya version of the epic Mahabharata. Apparently, Lord Krishna appeared in Nabagunjara form consisting of the head of a cock, throat of a peacock, tail in the form of a serpent, waist of a lion, hump of a bull, a leg each of horse, elephant, tiger and human to fool his friend Arjuna. The Chimera was holding a lotus flower in the human hand. Arjuna had never seen such a creature in his life and guessed that this could not be a real animal but a form assumed by Lord Krishna and immediately bowed down at his feet. It is said that the human hand with the lotus provided the clue. In the paintings and sculptures however, the lotus is often replaced by a "Chakra" or the "stylized discus" of Lord Krishna. Chimeric forms are encountered in literature and art all over the world. However, a chimera of nine animals is uniquely Orissan. Therefore, it was considered to be an appropriate emblem for the Journal of the Zoological Society of Orissa.

K Bohidar
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OCCURRENCE OF GONDWANA ANIMAL/ICHNO FOSSILS IN TALCHER BASIN, ORISSA AND THEIR SIGNIFICANCE

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ABSTRACT

Record of Gondwana animal/ichno fossils in different lithological formations of Talcher Basin, Orissa are discussed and analysed in this article. Few animal fossils (insect wing, annelids) are recorded only in Talchir Formation of this basin. Except Karharbari Formation ichno fossils are recorded in all other Gondwana formations of Talcher Basin. Earlier Lower Gondwanas of Talcher Basin were considered entirely of fresh water deposits. However, the marine nature of the Talcher, Barakar, Barren Measures and Kamthi sediments of this basin was predicted on the basis of typical marine ichnofossils. Hence, the previous model of continental facies for the Lower Gondwanas of Talcher Basin is found to be incorrect. Thus, the ichnofossils (*Skolithos* and *Cruziana* ichno-facies) of marine origin can be utilized as a tool for palaeo-environmental reconstruction. Here in Talcher Basin, marine incursion could have occurred due to the well known global transgressions during Permian and Triassic time. These evidences of signature for marine environment demonstrate a parallel (coastal marine to deltaic) mode of origin of the Gondwana coal beds and associated sediments.

Key words: Talcher Basin, Gondwana, animal/ichno fossils, marine incursion

INTRODUCTION

The Talcher Basin constitutes the southeastern most member of the Mahanadi Master Basin and occupies an area of over 1813 sq km within the Dhenkanal and Angul Districts along with a small portion of the adjoining Sambalpur District. This basin mainly occupies the Brahmani River Valley. The basin is bounded by latitudes 20°50 ' and 21°15 ' N and longitudes 84°09 ' and 85° 33 ' E (Fig. 1).

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Footprints, trails, burrows and tubes, coprolites and fossilized eggs are termed as ichnofossils (also known as trace fossils). Report of animal/ichnofossils in different formations of Talcher Basin and their diversity are discussed in the present study.

Geological Setting

This basin depicts a northwesterly plunging synclinal structure with closure to the east and younger horizons outcropping towards the west. The bed dips to the north and the number of coal seams increases in that direction, indicating a possible homoclinal structure. The Gondwana-Precambrian boundary in the North is marked by a WNW-ESE trending set of faults, but the southern boundary is void of any major faulting. This basin is marked by three sets of intra-basinal faults trending E-W, NE-SW and WNW-ESW. Although dips are usually shallow in this basin, steeper gradients have been encountered near faults. The regional strike of the Gondwana sedimentary rocks of this basin is more or less East-West, but varies from ENE-WSW to ESE-WNW (Raja Rao, 1982; Manjrekar et al., 2006; Goswami et al., 2006a, b; 2007). Stratigraphic nomenclature of Talcher Basin is given in Table 2.

Animal/ ichnofossils

The use of ichno fossils in the study of palaeoenvironment and sedimentology has certain distinctive advantages over other fossils, as they are mostly autochthonous and commonly found in deposits where other fossils are rarely preserved and are not easily visible. There is a close relationship between trace fossil assemblages, sediments and environment model (Mukhopadhyay, 1996). The common association of some trace fossils and its recurrence in stratigraphic sequence of coal bearing Gondwana sediments may be comparable to well-established ichno-facies of Seilacher (1964, 1977, 1978).

Sparse bioturbation in Lower Barakar sediments of Talcher and Ib River basins with few identifiable trace fossils like *Palaeophycus* and shallow burrows of insects (De, 1990), which are generally associated with fireclay and root beds, could be classified under continental *Scoyenia* ichno-facies (Mukhopadhyay, 1996).

Vertical trace fossils like *Skolithos, Arenicolites, Diplocraterion, Corophioides, Teichichnus, Rosselia, Psilonichnus* etc. indicate the characteristic behavioural patterns of animals in high energy environment, where the ecological stress compels the animal to make deep burrows and reinforce the structure by lining with mucous, mud or on particles. The universal presence of

burrow lining and wave ripple lamination within the host sediments clearly testify that these trace fossils belong to marine *Skolithos* ichno-facies (Mukhopadhyay, 1996).

Inclined to horizontal burrows such as *Rhizocorallium, Phycodes, Planolites,* trails of *Scolicia* and other types, indicate the activity of animals in an environment lacking vigorous current and wave activity occurring below the wave base. These trace fossils generally belong to marine *Cruziana* ichno-facies of Seilacher (1964, 1977, 1978).

It is important to note that both *Skolithos* and *Cruziana* ichno facies are found in good numbers in the Permian sediments of the Talcher Basin. Prolific occurrence of the ichnogenera such as *Cylindrichnus, Rosselia, Teichichnus, Rhizocorallium, Skolithos, Psilonichnus* etc. (belonging to *Skolithos* and *Cruziana* ichno-facies) (Table 1) in this basin are indicative of a shallow-marine environment (Mcarthy, 1979; Pollard, 1988).

Ichnofossils ranging in age from Early/Lower Permian to Late/Upper Triassic were collected in the Talcher Basin from various localities and different formations (Srivastava et al., 1996 and De, 1998; 1999 a; 1999 b; 2001).

Talchir Formation (early Early/Lower Permian)

Talchirichnus gondwanensis, an animal trail has been reported from the rocks of the Talchir Formation in an outlet of Nandir Jhore stream, near Teheranpur Village by Srivastava *et al.* (1996). De (1998; 1999 b) reported thirteen more ichnotaxa from a outcrop section, Angul-Kosala road of Talchir Formation of this basin such as Bivalve trails, Bivalvian resting traces, feather stitch trails, *Furculosus,* gastropod trails, *Granularia, Nereites, Pelecypodichnus, Planolites, Rhizocorallium, Sabellarifex, Scalarituba* and *Skolithos.* Altogether 14 ichnotaxa at the species level have been reported from this formation in the Talcher Basin (Table 1).

Chandra and Singh (1996) reported more plant and animal fossils from the Type Locality of Talchir Formation exposed near Sarang Village, Angul District. The fossils include few bryophytic remains, four species of *Gangamopteris*, one species of *Noeggerathiopsis*, equisetalean leaflets, equisetalean stems, two fertile organs i.e. *Ottokaria bengalensis* and *Arberia surangei*, impressions of nematodes, annelids and an insect wing. This is the first record of plant/animal co-existence in the earliest Permian Talchir rocks in India.

Barakar Formation (late Early/Lower Permian)

De (1998; 1999 b) studied 12 ichnotaxa such as *Chondrites, Cylindrichnus, Ophiomorpha, Planolites, Psilonichnus, Rosselia, Skolithos, Teichichnus, Thalassinoides, Terebellina, Zoophycos* and spiral gastropod burrows from the Barakar Formation of this basin (Table 1). These ichnofossils are reported from three outcrop sections (one in Angul-Kosala road, another two in east and southwest of Kosala) and one drill core (Borehole II at Mamuria) of the Barakar Formation of this basin.

Barren Measures (Middle Permian)

De (1999 a) reported four ichnotaxa (*Monocraterion, Planolites, Psilonichnus* and *Skolithos*) from the outcrop sections of Barren Measures Formation around Brahmani River Valley of this basin (Table 1).

Lower Kamthi (Late/Upper Permian) and Upper Kamthi (Triassic)

De (1999a) recorded two ichno-genera, *Chondrites* and *Planolites* from the outcrop sections of the Lower Kamthi Formation around Brahmani River Valley. *Skolithos* and *Thalassinoides* (two ichnotaxa) are recorded from the same outcrop sections of Upper Kamthi Formation by De (1999a) (Table 1).

Thus, a complete ichno-stratigraphic account comprising 19 ichno genera along with various ripple surface-bound trails of bivalves and gastropods, bivalve-resting traces and spiral gastropod burrows has been reported from Lower Kamthi exposures of the Talcher Basin (Table 1).

DISCUSSION AND CONCLUSION

Hence, the study demonstrates that the trace fossils including various types of feeding and escape traces occur in almost all the Gondwana formations (except Karharbari) of Talcher Basin of Orissa with striking density and diversity suggesting the high ichnological potential of the sediments.

The present status warrants the need for more systematic ichno-stratigraphic analysis of this basin to reconstruct a complete ichnological model of palaeoenvironment and to understand the extent of marine influences in continental coal-bearing Gondwana deposits.

Miller (1984) inferred that non-marine animals capable of making deep burrow generally did not exist before Mesozoic. The presence of *Skolithos* and *Cruziana* ichno-facies with well developed lining on the wall of deep burrow greater than 1 cm in length in different levels of Gondwana (Palaeozoic-Mesozoic) sediments in Talcher Basin, which are commonly regarded as characteristic ichno-facies of typical shallow marine environment (Mcarthy, 1979). It depicts that the coal measures of these basins were deposited in tidal flat to shelf environment (Mukhopadhyay, 1996). The observed uniformity in stratigraphic distribution of different ichno-genera firmly suggests parallel palaeo-environmental development characterized by frequent shallow marine events. It also reveals that short-lived marine incursions, splitting of coal seams at places into transgressive-regressive couplets and strong in-faunal activities might have occurred in the Talcher Basin during respective time (De, 2001; Goswami, 2002). All the above-mentioned ichno-forms are comparable to the marine-influenced lower deltaic plain ichno-faunas of the Pennsylvanian of United States (Archer and Maples, 1984) and marine Permian Gondwana ichno-taxa of South Africa (Hobday and Tavener Smith, 1975; Masson and Christie, 1986; Kamola, 1984; Rhoads, 1975). High bioturbation, diversity and abundance of trace fossils are most common in Talchir Formation of Talcher Basin and decrease towards the upper part of the formation. Proliferation of trace fossils is observed in sandstones and siltstones of different Lower Gondwana formations. Common occurrences of sequential bioturbation signify fluctuation in rate of sedimentation and energy of the environment. The lithic make-up of these formations along with its diverse ichnofauna and high rate of bioturbation, positively indicate the presence of shallow-shelf environment during the deposition of coal-bearing Gondwana sediments (Mukhopadhyay, 1996).

The above ichnological evidences reflect a parallel (coastal marine to deltaic) mode of origin of the Gondwana coal beds and associated sediments in the Talcher Basin, Orissa (De, 2001; Goswami, 2002). The marine influence is further supported by the findings of marine Stromatolites and entombed marine cyanophytes from Talchir Formation of Talcher Basin at and around Bedashar Village, Angul District (Pandya, 1995; De, 1999b).

It is observed that the trace/ichnofossils are more common within the bioturbated sequence of different Permian formations. It is evident that the higher the degree of bioturbation, the lower the rate of sedimentation and physical energy condition (Howard and Frey, 1984). Thus, the animals responsible for traces possibly thrived on the bottom sediments but could not undergo burial due to low rate of deposition. It is quite likely that they became victims of

large-scale destruction by predators and scavengers in this basin (Bobde, 1979; Srivastava et al., 1996; Goswami, 2002).

The diversity of animal and ichnofossils in Talcher basin is plotted in Fig. 2. In the Talcher Basin, the diversity of ichno and animal fossils has reached at its acme during early Early/ Lower Permian time (during the deposition of Talchir Formation) (16 species) and followed by late Early/Lower Permian time (during the deposition of Barakar Formation) (12 species), Middle Permian time (during the deposition of Barren Measures Formation) (4 species), Late/Upper Permian time (during the deposition of Lower Kamthi Formation) (2 species) and Triassic time (during the deposition of Upper Kamthi Formation) (2 taxa). No ichno taxa are reported from Karharbari sediments from this basin.

Table 1. Comparative analyses of Gondwana ichno taxa in Talcher basin of Orissa

	Talcher Basin					
Ichno/ animal taxa	Talchir Formation (Lower Permian)	Karharbari Formation (Lower Permian)	Barakar Formation (Lower Permian)	Barren Measures Formation (Middle Permian)	Lower Kamthi Formation (Upper Permian)	Upper Kamthi Formation (Triassic)
Animal fossils	2	0	0	0	0	0
Annelids	+					
Insect Wing	+					
Ichnofossils	14	0	12	4	2	2
Bivalve trails	+					
Bivalvian resting traces	+					
Chondrites			+		+	
Cyclindrichnus			+			
Feather stitch trails	+					
Furculosus	+					
Gastropod trails	+					
Granularia	+					
Monocraterion				+		
Nereites	+					
Ophiomorpha			+			
Pelecypodichnus	+					
Planolites	+		+	+	+	
Psilonichnus			+	+		
Rhizocorallium	+					
Rosselia			+			
Sabellarifex	+					
Scalarituba	+					
Skolithos	+		+	+		+
Spiral gastropod burrow			+			
Talchirichnus gondwanensis	+					
Teichichnus			+			
Terebellina			+			
Thalassinoides			+			+
Zoophycos			+			
Total taxa	**16**	**0**	**12**	**4**	**2**	**2**

Table 2:Stratigraphic nomenclature of Talcher basin, Orissa (after Manjrekar et al. 2006; Goswami et al. 2006b; 2007)

Age	Formation/ Member	Lithology and fossil content	Thick-ness
Recent		Alluvium and laterite	
Upper Triassic Lower Triassic	Upper Kamthi	Upper bed (Upper Triassic): Ferruginous, hard and quartzitic sandstones , bands of compact brown, grey and yellow shales and clasts of creamy white shales. Lower bed (Lower Triassic): Medium-grained, crossbedded ferrugineous yellowish white sandstones, alternating with thick bands of red and grey shales.	250 + meters
Upper Permian	Lower Kamthi	Medium to coarse grained, pebbly cross-bedded ferruginous sandstones, clasts of greenish-white and grayish-white shales, pink clays	
Middle Permian	Barren Measures	Coarse to medium grained greenish grey feldspathic sandstones with shreds and lenses of chocolate coloured clay, micaceous siltstone, dark grey shale, carbonaceous shale, purple brown shale and clay ironstone.	317+ meters
Late Lower Permian	Upper Barakar	Fine to coarse grained feldspathic whitish sandstones, siltstone, grey shale, sandy shale, fireclay and coal seams with polymictic conglomerate at the base.	600 meters
Late Lower Permian	Lower Barakar	Arkosic, coarse to granular to pebbly sandstone with minorgrey shale and siltstone, carbonaceous shale and coal seams.	
Middle Lower Permian	Karharbari	Medium to Coarse grained whitish arkosic sandstones, carbonaceous shale, grey shale and coal seams with polymictic conglomerate at the base.	270 meters
EarlyLower Permian	Talchir	Diamictites, rhythmites, turbidites, conglomerate, fine to medium-grained greenish sandstones, olive coloured needle shales, turbidite, tiliets and tilloids etc.	170 meter +
		unconformity	
Precambrian		Granites, gneisses, amphibolites, migmatites, quartzite and pegmatites	

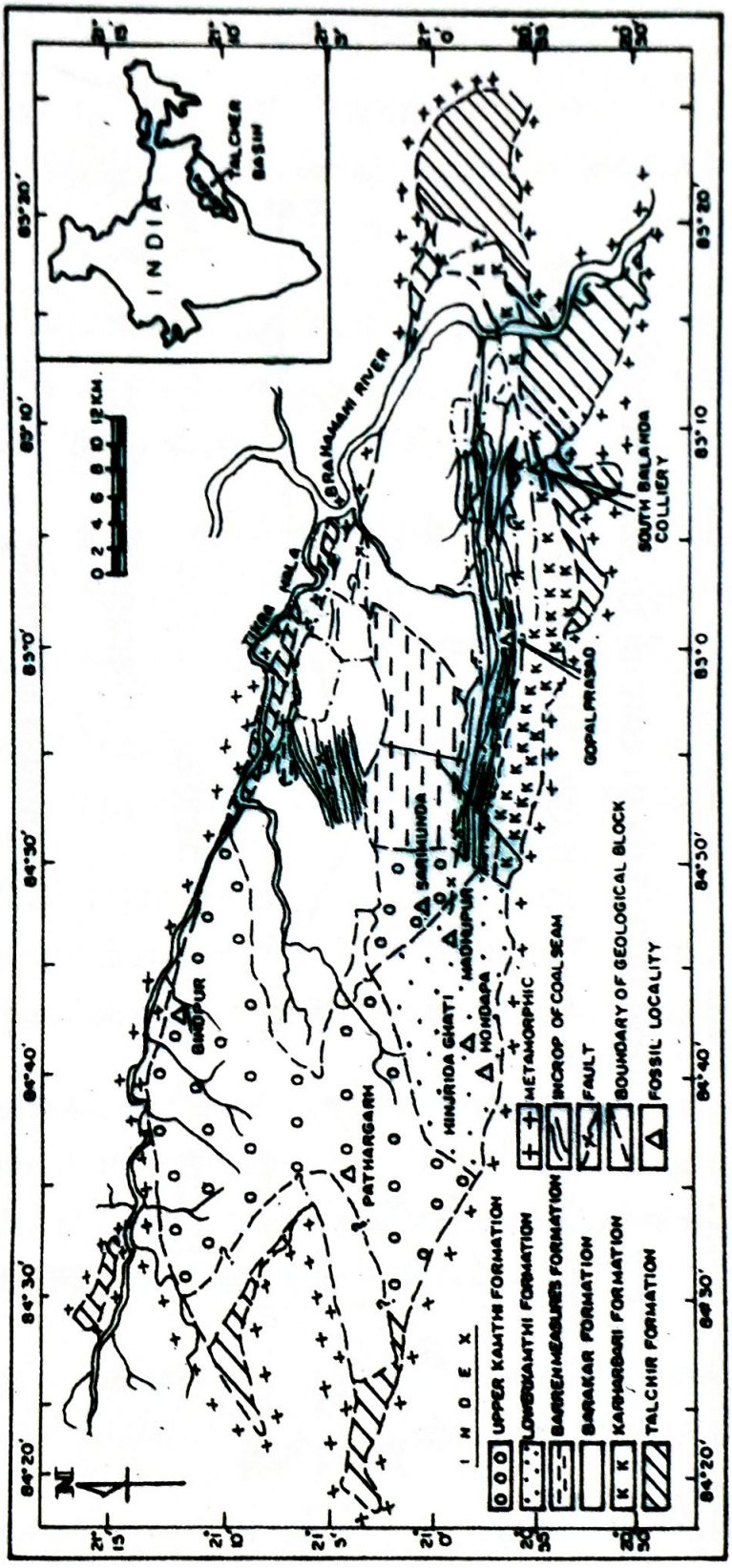

Fig. 1: Geological map of Talcher Basin, Orissa (Modified after Manjrekar *et al.*, 2006; Goswami *et al.*, 2007)

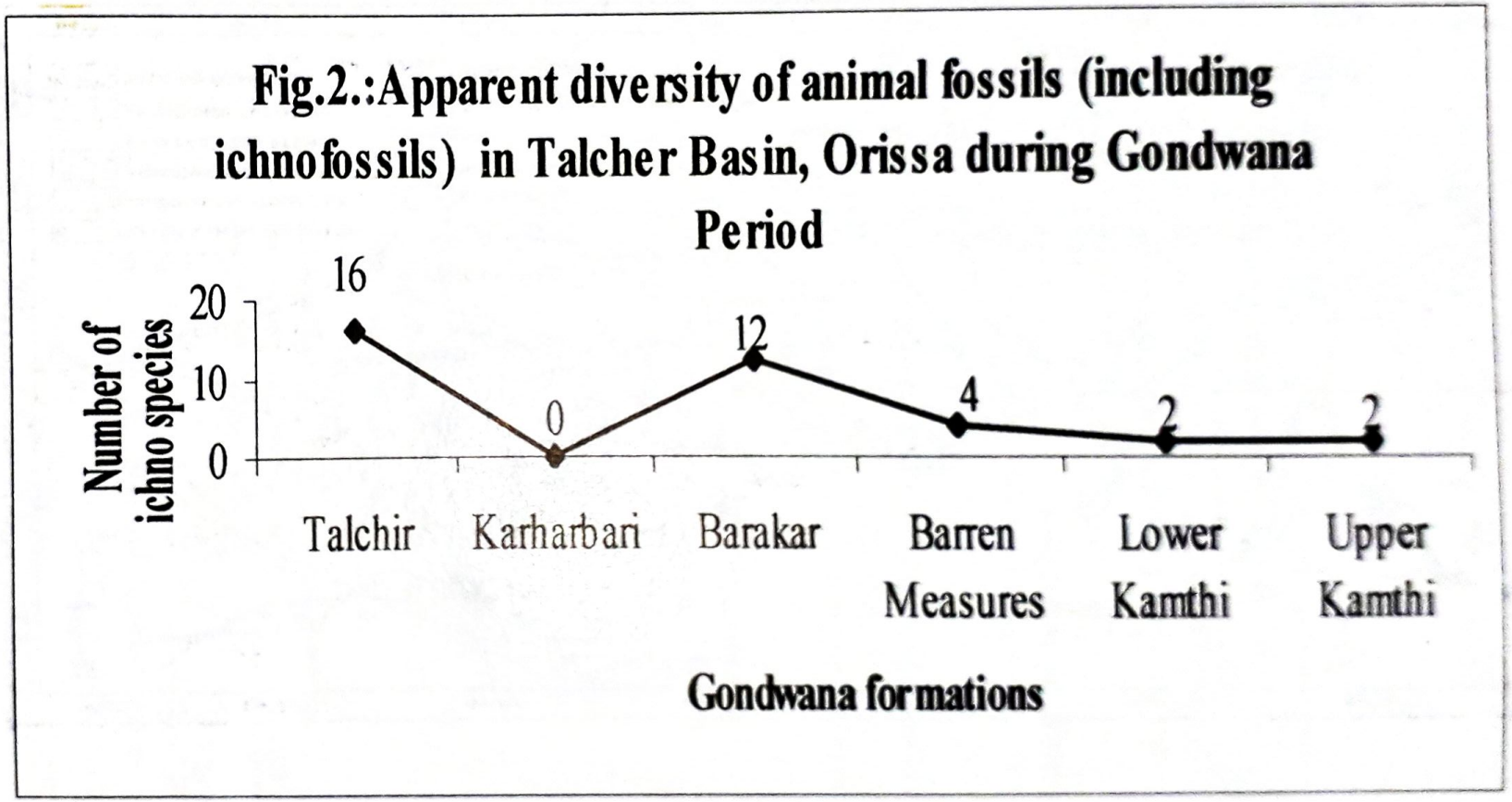
Fig.2.:Apparent diversity of animal fossils (including ichnofossils) in Talcher Basin, Orissa during Gondwana Period
Number of ichno species
20
10
0
16
0
12
4
2
2
Talchir
Karharbari
Barakar
Barren Measures
Lower Kamthi
Upper Kamthi
Gondwana formations

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WHY WASTE POULTRY VISCERA?

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ABSTRACT

The by-products of poultry industry are a rich source of protein and proteolytic enzymes. In the present paper a standardize procedure to retrieve proteins in the form of protein hydrolysates from chicken intestine has been described.

Key words: Poultry viscera, protein hydrolysate

INTRODUCTION

By-products of poultry industry, which include viscera, bone, blood, head, feet and feather, together constitute 28-30% of the total weight (Panda and Singh, 1980; Ockerman and Hansen, 2000). Intestine, which accounts for 20-30% of this waste (Panda and Singh, 1980) is a potential source of proteins lipids (Rao et al., 1996, Ockerman and Hansen, 2000) and tissue proteases (Raju et al., 1997). However, this commodity has not found favor with consumers, mainly due to aesthetic and hygienic reasons. Hence, presently, the bulk of this waste is being either discarded or partly used in animal feeds. In India, poultry industry is growing steadily and is estimated to produce annually more than 65,000 tons of intestine (Rao et al., 1996). Although some attempts have been reported for preparing hygienised animal feed products from chicken intestine (Giri et al., 2000), no systematic attempt seems to have been made to utilize proteins and proteolytic enzymes from this valuable by-product. One of the major objectives of our laboratory is to standardize suitable procedures to retrieve proteins from chicken intestine using tissue autolysis. The present paper reports kinetics of interaction of tissue proteins with endogenous proteases, which yields methods for i) retrieving proteins in the form of protein hydrolysates and ii) for reducing the bitterness associated with commercially protein hydrolysates using chicken intestine exopeptidases.

MATERIALS AND METHODS

1.Enzyme assays:

The activity of aspartic protease was determined according to Barrett (1970) using Bovine Haemoglobin type II [Hb] (Sigma Chemical Co., St. Louis, MO, USA) as substrate at pH 3.0

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and 50°C. The TCA soluble products were determined according to the method of Lowry modified by Miller (1959).

Aminopeptidase activity was determined by the method of Barrett (1972) using d β Naphthylamine derivative of amino acids at 50°C and pH 6.8. The pink color developed by adding Fast Garnet GBC (in 4% Brij 35) and 10 mM PCMB reagent mixed equally just before terminating the assay was measured at 520 nm.

2. Preparation of protein hydrolysate (PVPH) from poultry viscera:

The autolytic method used for the preparation of protein hydrolysate is outlined in Fig. 1. A 40% homogenate was prepared in distilled water by grinding the tissue in blender (Sumeet, Mumbai, India). The pH of the homogenate was adjusted to 2.8 by adding 1N HCl and incubated at 55°C for 6h with constant stirring at 150 rpm using overhead stirrer (DBK instruments, Mumbai). Samples were withdrawn at different time points during the hydrolysis reaction. Heat inactivated (90°C for 15 minutes) homogenate (pH 2.8) incubated under similar conditions served as unautolysed control. After 6 hours the samples were centrifuged at 20,000g for 15 minutes and the supernatant was dried in a vacuum drier at 60°C to obtain the protein hydrolysate. The product was analyzed for proximate composition as well as for functional properties.

Debittering of protein hydrolysates by immobilization method

3. Separation of Mucosa:

Chicken intestine was brought in ice from local abattoir soon after slaughter and freed of accompanying organs such as spleen and pancreas along with the overlaying fat and other connective tissue. The undigested food and faecal matter were flushed out with tap water. The intestines were cut open longitudinally; mucosa was scraped out, packed in polythene bags and irradiated (20 kGy) in Gamma Cell (AECL, Canada).

Preparation of Beads: The immobilized beads were prepared by entrapping the irradiated (20 kGy) mucosa (20 % v/v) in 3% Sodium Alginate (Loba Chemicals, India) and adding this solution dropwise into 0.3 M $CaCl_2$. The beads were left overnight in $CaCl_2$ for hardening and washed with distilled water the next day.

Debittering of protein hydrolysates:

The scheme for debittering protein hydrolysates with immobilized chicken intestinal mucosa is presented in Fig.2. The calcium alginate beads entrapped with mucosa (30 g) were packed in a jacketed column (43 cm x 1.5 cm) maintained at 50°C. The bitter protein hydrolysate was passed through the column at a flow rate of about 45 ml/hr. The solution eluting from the column was collected. The debittering of the solution was assessed by taste panel as well as RP- HPLC analysis.

RESULTS AND DISCUSSION

Proteolytic Profile:

Chicken intestine possesses high levels of cathepsins, aminopeptidases and alkaline proteases (Table 1). The specific activities of the enzymes in intestinal tissue were 1.5 to 3 fold higher than that in skeletal muscle, liver and spleen.

Table 1. Levels of proteases in chicken tissues.

Enzyme	Specific activity (nmoles/mg protein /h)			
	Liver	Spleen	Skeletal Muscle	Intestine[a]
Cathepsin B	31.5 ± 0.30	22.1 ± 0.37	1.9 ±0.11	69.5 ± 0.78 (48%)
Cathepsin D	2230 ± 60.00	2260 ± 24.00	73 ± 2.00	4700 ± 37.00(53%)
Cathepsin H	110 ± 3.90	93.2 ± 1.23	17.2 ±0.54	310 ± 4.68(39%)
Cathepsin L	631.5 ± 30.25	412.0 ± 35.00	83.6 ± 14.50	843.9 ± 61.00(27%)
Phe-Arg aminopeptidase	179.5± 7.40	198.5 ± 1.14	6.4 ± 0.36	408.8 ± 8.24 (34%)
Ala aminopeptidases	81.6 ± 2.90	49.4 ± 4.33	11.6 ± 0.15	169.2 ± 4.17(66%)
Leu aminopeptidases	69.9 ± 4.30	63.3 ± 1.81	9.1 ± 0.14	165 ± 8.03 (36.6%)
Pro aminopeptidases	22.4 ± 1.30	15.7 ± 0.734	5.7 ± 0.77	46 ± 2.62(34%)
Tyr aminopeptidases	63.7 ± 2.00	46.9 ± 2.53	8.5 ± 0.53	190.9± 14.52(38.5%)
Alkaline Proteases	126 ± 15.60	213.7± 12.61	28.7 ± 1.60	1346 ± 10.30 (64%)

[a] Values in parenthesis represent the yield of the enzymes in the intestine, considering the sum of the activity associated with all the four tissues, as 100%.

Assuming that these enzymes in chicken are distributed largely among liver, spleen, skeletal muscle and intestine, the yield in intestine could be estimated as 36-64% (Table 1 parenthesis). The protein content of the intestine calculated in terms of dry weight of the tissue was 57-60%. Chicken intestine being a good source of both proteins and proteolytic enzymes, the possibility of autolytic degradation of proteins becomes strong, which in turn, could serve as a means to retrieve value added protein products from this otherwise underutilized source. The high levels of aminopeptidase activity suggest the possibility of using them for the debittering of protein hydrolysates.

Fig.1 Preparation of chicken intestinal protein hydrolysate:

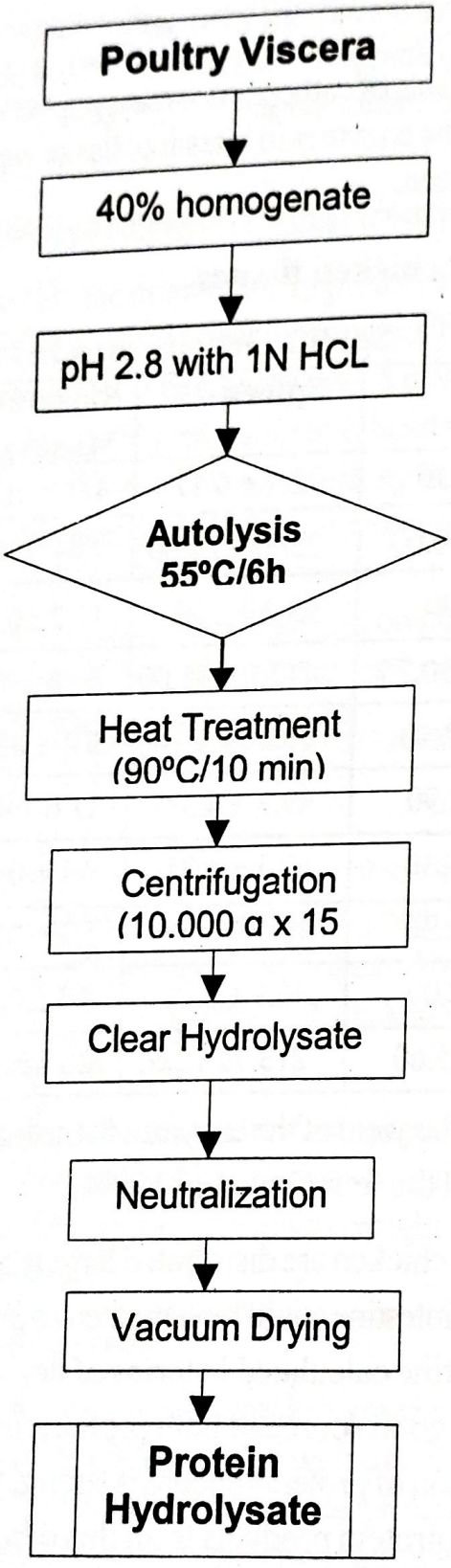
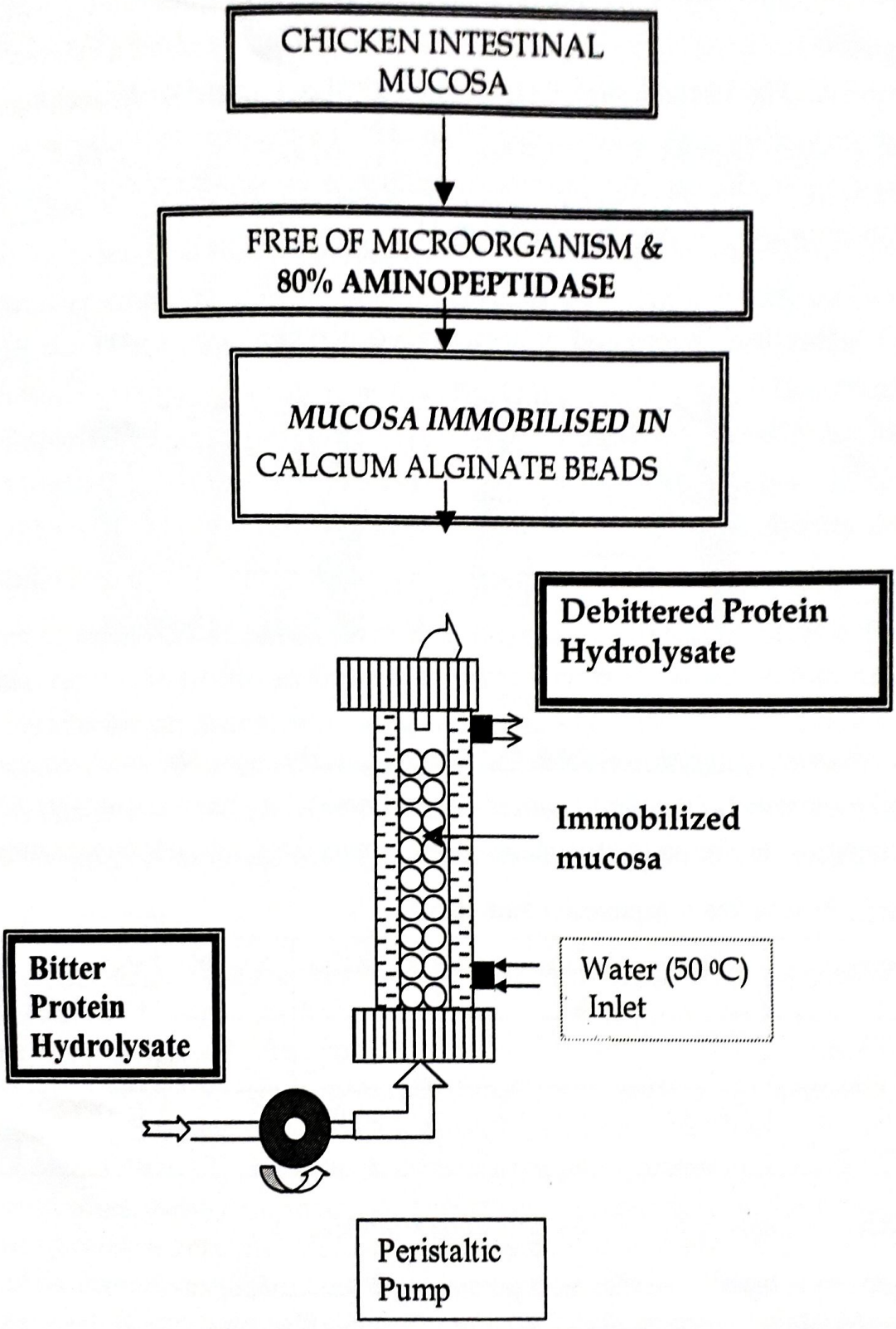


Fig. 2 Scheme for debittering of protein hydrolysate:



Preparation of chicken intestinal protein hydrolysate:

A rapid procedure for recovery of proteins in the form of hydrolysate from poultry viscera using tissue autolysis (Fig.1) is reported. The method yields a light brown, hygroscopic protein hydrolysate (Fig. 3B) with superior microbiological quality and functional properties. The conditions used for tissue autolysis (pH 2.8 and 55°C) were optimized earlier in our laboratory (Jamdar and Harikumar, 2005) and the enzymes responsible were identified as endogenous aspartic proteases like pepsin or cathepsin D.

Fig. 3 A. Poultry Viscera and B. Protein Hydrolysate

A B

The proximate composition (Table 2) shows that nearly 52% of the total tissue dry matter could be retrieved in the form of protein hydrolysate with a recovery of 80% of the tissue protein nitrogen. The product contained 84% protein, 6.5% ash and 9% moisture.

Table 2. Proximate composition and yield[a]

Sample	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)	Yield (%)	
					Dry matter	Nitrogen
Poultry Viscera	76.5 ± 1.50	12.8 ± 1.2	6.9 ± 1.25	1.1 ± 0.23	-	-
Protein hydrolysate	8.9 ± 2.4	84.0 ± 6.4	0.3 ± 0.08	6.5 ± 1.2	52.0 ± 2.4	80.2 ± 2.1

[a] *Values are means ± SD of triplicate determination.*

Our process is superior to other methods employed for the retrieval of proteins from chicken (Surowka and Fik, 1992; Surowka and Fik, 1994) and fish (Shahidi et al.,1995) processing

wastes mainly due to increased recovery of protein nitrogen. Moreover, unlike other methods (Surowka and Fik ,1992; Surowka and Fik,1994; Shahidi et al.,1995) our autolytic procedure is cost effective since no exogenous proteases are used for the hydrolysis of proteins.

Debittering of Protein hydrolysate:

The method developed by us for the debittering of protein hydrolysates using chicken intestinal mucosa is presented in Fig.2. Entrapment in Ca-alginate served as an easy method for the immobilization of mucosal aminopeptidases. Aminopeptidase in the immobilized system exhibited broader temperature and pH optima than in the free form. Debittering of protein hydrolysates was effected by a single pass of the hydrolysate through the immobilization system. Flow rate was maintained to allow a contact time of approximately 1h between the hydrolysates and the beads. The bitterness of protein hydrolysates is generally ascribed to peptides (<10 kDa) rich in hydrophobic amino acids in certain specific sequences. The action of exopeptidases to bring about a reduction in bitterness of protein hydrolysates, is well known (Raskulthai and Haard, 2003).

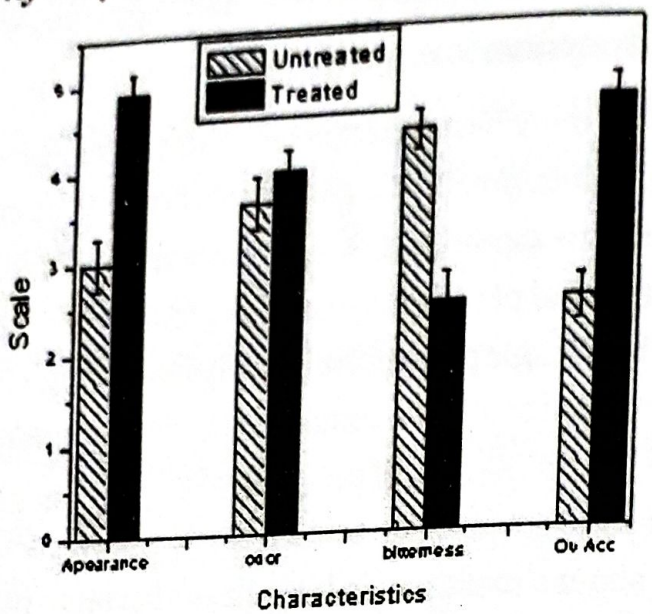
The sensory characteristics of debitered protein hydrolysates are presented in Fig.4. Protein hydrolysates prepared from both casein as well as soyabean proteins showed increase in overall acceptability after enzyme treatment, accompanied by distinct decrease in bitterness. (from 4.30 to 2.40 for casein hydrolysate, (Figure 4A) and from 3.8 to 2.40, (Figure 5B) for Soybean hydrolysate. The taste panel studies also convincingly proved the reduction in the bitterness of the protein hydrolysates after treatment with chicken mucosal peptidase acted on them.

Continuous operations:

The effect of continuous operations on the debittering activity and enzyme retention was monitored. In all the aliquots collected, a general shift in the peptide peaks from 25- 45 min to 3-22 min was observed with little leakage of activity during the process. Non availability of catalytically efficient peptidases in economically viable quantities is the major limitation faced by industries attempting enzymatic debittering of protein hydrolysates. Since different types of amino acids are involved in imparting bitterness to protein hydrolysates, it is desirable to use a process, which uses a group of enzymes with varied specificities. In this respect, our process using chicken intestinal mucosa which is endowed with advantages such as the presence of multiple exopeptidases capable of acting on all the amino acid residues, high rates of catalytic efficiency and stability over a wide range of temperatures and pHs encountered in food industry operations.

Fig. 4. Sensory analysis of protein hydrolysate.

A: Casein hydrolysate



B: Soybean protein hydrolysate

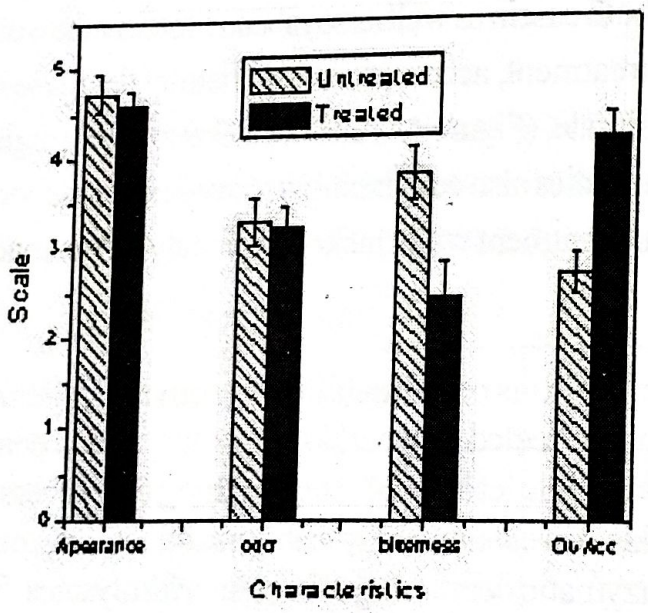
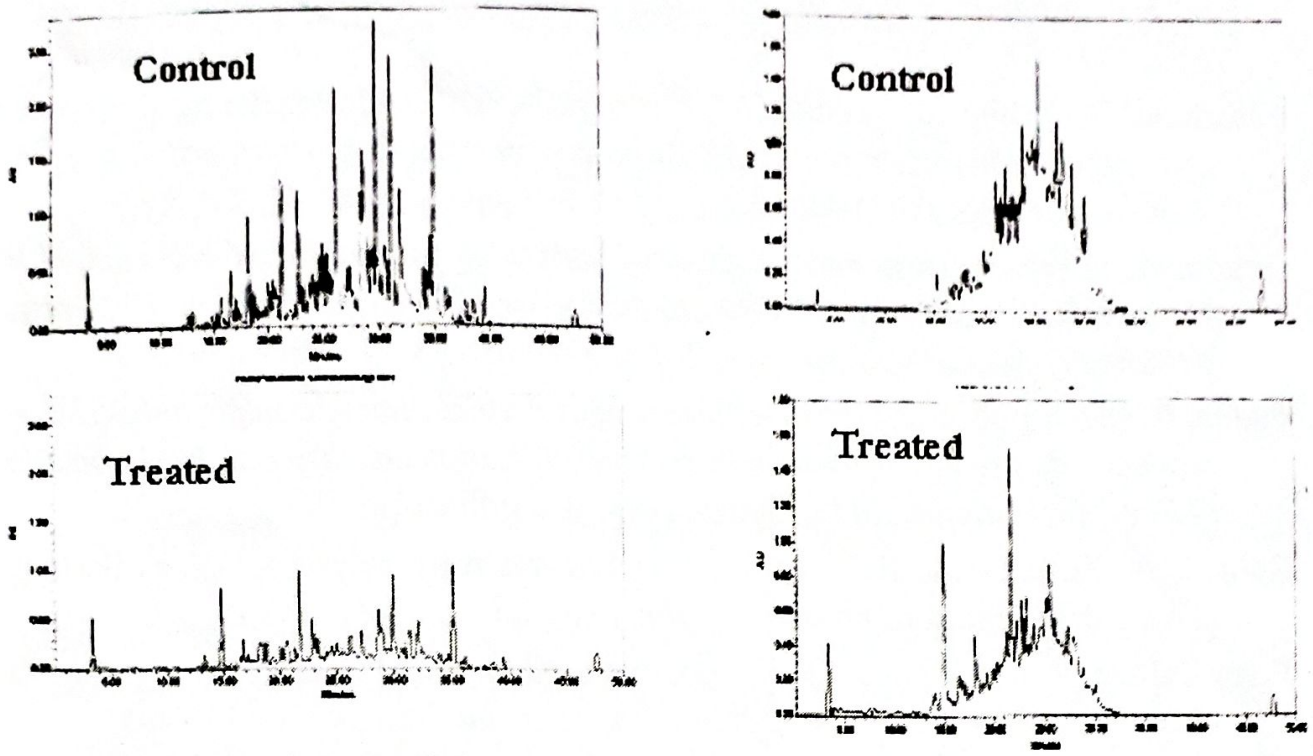


Fig. 5: RP HPLC profiles of protein hydrolysates after debittering.



Casein Hydrolysate (A) Soy Protein Hydrolysate (B)

The methods described in this paper could be effectively used to retrieve proteins as well as proteolytic enzymes from intestine, an underutilized waste of poultry processing industry.

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HEMATOLOGICAL AND BIOCHEMICAL STUDIES OF FAN-THROATED LIZARD, *SITANA PONTICERIANA* OF ORISSA, INDIA (SAURIA: AGAMIDAE)

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ABSTRACT

Present study deals with hematological and biochemical parameters of Fan-throated lizard (*Sitana ponticeriana,* Cuvier, 1829) collected from Balukhand-Konark area of Orissa. Total erythrocytes count (TEC), total leucocytes count (TLC), hemoglobin content (Hb), differential leucocytes count (DLC) in %, length, breadth and size of different blood cells, space occupied by nuclei and cytoplasm were studied. Amount of protein and cholesterol in the blood serum was also a part of investigation.

Key words: *Sitana ponticeriana,* Hematology, Blood serum.

INTRODUCTION

Haematology and serum chemistry play an important role in determining the health condition of reptiles (Campbell, 1998; Martinejz- Silvestre et al., 2004). The haematological parameters of healthy reptiles are influenced by a number of internal and environmental factors (Lillywhite and Smits, 1984; Frye, 1991; Anderson, 1992; Barten, 1993). Even though there are several reports on haematology and serum chemistry of different reptiles (Saint Girons, 1970; Duguy, 1970; Alleman et al, 1999; Sevinc et al., 2000), many reptiles are not yet studied because of their limited accessibility. Moreover, reptiles are highly heterogenous class in which it is difficult to draw inference from one specimen to other (Martinejz-Silvestre, 2005). It is therefore

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important that the classical characteristics of blood cells of each species are known. Establishment of haematological and blood biochemical values may be useful in wild life rehabilitation center (Raphael et al., 1994). Also monitoring reptile blood parameters can be a way to manage and evaluate the physiological and health status of their populations and may be a useful indicator of the environmental status, because reptiles are very sensitive to habitat change (Dickinson et al., 2002).

In India, hematological studies are confined to humans and some economically important animals. Studies on reptiles of Orissa (India) are usually restricted to morphology and systematics. So, in the present study various hematological and biochemical parameters of the fan-throated lizard *Sitana ponticeriana* have been described for the first time. This species is selected because of its hardiness and relative popularity in natural habitat.

MATERIALS AND METHODS

Blood samples were gathered from healthy and active *Sitana ponticeriana* from Chandrabhaga, Balukhand-Konark area, Orissa, India 19°51' 35.99" N, 86°05' 13.78" E. Ten specimens of each sex were collected for this purpose. Before collection of blood, specimens were weighed and their snout-vent length (SVL) and tail length (TL) were measured. Samples were obtained manually by veinipuncture using a lateral approach to the coccygeal vein. Blood smears were obtained using push slide technique and stained with Giemsa's stain. Sahli's hemometer was used for estimation of haemoglobin and for counting the number of RBCs and WBCs, following standard procedure and cell numbers were counted with the help of a haemocytometer under compound microscope. The RBC, WBC and its nuclei were measured by an ocular micrometer, which was standardized against a stage micrometer (ERMA, Japan make). The size of the RBCs and their nuclei were obtained measuring their long and short axis (length and breadth) in case of elliptical RBCs. For rounded RBCs only one axis (diameter) was considered. Protein content of blood serum was determined according to the method of Lowry et al., (1951) and cholesterol content of blood serum was determined according to the method of Rosenthal, (1960).

RESULTS

Ten adult males and ten females were collected for the investigation. The males weighed from 3.4 to 4.5g (3.95 ± 0.55) where as females weighed 2.6 to 3g (2.8 ± 0.2), respectively. Body size (total length = TOL) of males ranged from 15.2 to 15.8 cm (15.5 ± 0.3) whereas females ranged from 12.8 to 13.2cm (13 ± 0.2), respectively.

Morphology and morphometry of blood cell (Table 1, 2 and Fig. 1-2)

A. Erythrocytes or Red Blood Corpuscles (RBC).

The RBCs were elliptical in shape with oval nucleus. Sometimes RBCs were rounded with rounded nuclei. In Giemsa's stain the cytoplasm of these RBCs appeared to be light violet in colour. In some other cells the nucleus looked deep pink in colour. These RBCs were termed as normocytes since these were matured and inactive erythrocytes at the verge of extinction.

The long axis (i.e. length) of erythrocytes ranged from 17.94 to 19.32mm (18.85 ± 0.79mm) and the short axis (i.e. breadth) remained 11.04mm in males. In females it ranged from 20.07 to 22.08 mm with a mean long axis of 21.15 ± 0.79mm and the short axis (i.e. breadth) of erythrocytes ranged from 12.42 to 13.8mm with mean size (13.33 ± 0.79mm). The area occupied by the RBC ranged from 155.55 to 167.52mm^2 (163.44 ± 0.62mm^2) in males and it ranged from 195.77 to 387.96 mm^2 (221.42 ± 0.49mm^2) in females.

The length and breadth of nuclei was found to be 9.66 mm and 5.52 mm in males and in females it was11.04 mm and 6.9 mm respectively. The area occupied by the nuclei was 41.88 mm^2 in males and 59.82 mm^{2}in females. The ratio between cells and nuclei ranged from 3.71 to 4 (3.9 ± 0.1) in males and in 3.27 to 6.48 (3.70 ± 0.57) in females.

B. Leucocytes or White Blood Corpuscles (WBC)

The WBCs were of five types i.e. lymphocytes (large and small), monocytes, eosinophil, basophil and neutrophil. The first two types being agranulocytes while the rest were granulocytes.

i. Lymphocytes

The lymphocytes were rounded or spherical in shape. According to size, they were categorized as large and small lymphocyte. The nuclei were round in both large and small lymphocytes and occupied most of the entire cell. The large lymphocyte ranged from 13.8 to 16.56 mm with a

mean diameter of 15.18 ± 1.38 mm in males. In females it ranged from 15.18 to19.32 mm with a mean diameter of 47.47 ± 2.1 mm. The small lymphocytes were 8.28 to 11.4 mm in diameter (9.66 ± 1.38 mm) in males and in females they were 8.28 to 12.42 mm in diameter (10.11 ± 2.1 mm).

ii. Monocytes

Monocytes were found to be oval or rounded in shape with eccentric nucleus. Some nuclei were kidney shaped while others were elongated. The nucleus was deeply stained while the cytoplasm was lighter. The size of monocytes ranged from 13.8 to17.94 mm (15.63 ± 2.16 mm) in males and 13.8 to 16.56 mm (15.18 ± 1.38 mm) in females.

iii. Eosinophils

Eosinophils were identified by their large granular appearance. The granules were darkly stained. The nucleus was either two lobed or concentrated at one end of the cell, appearing to be thick and slightly notched in the middle. The cytoplasm was lightly stained. The size of eosinophils ranged from 8.28 to 11.04 mm in diameter (9.66 ± 1.38 mm) in males and 11.04 to 13.8 mm (12.42 ± 1.38 mm) in females.

iv. Basophils

Basophils were identified by the presence of granules over the nucleus as well as the entire cell. They were oval in shape and have a lobed nucleus. The size of basophils ranged from 8.28 to 9.66 mm with an average diameter of 8.73 ± 0.79 mm in males. In females it ranged from 4.14 to 5.52 mm with an average diameter of 4.59 ± 0.79 mm.

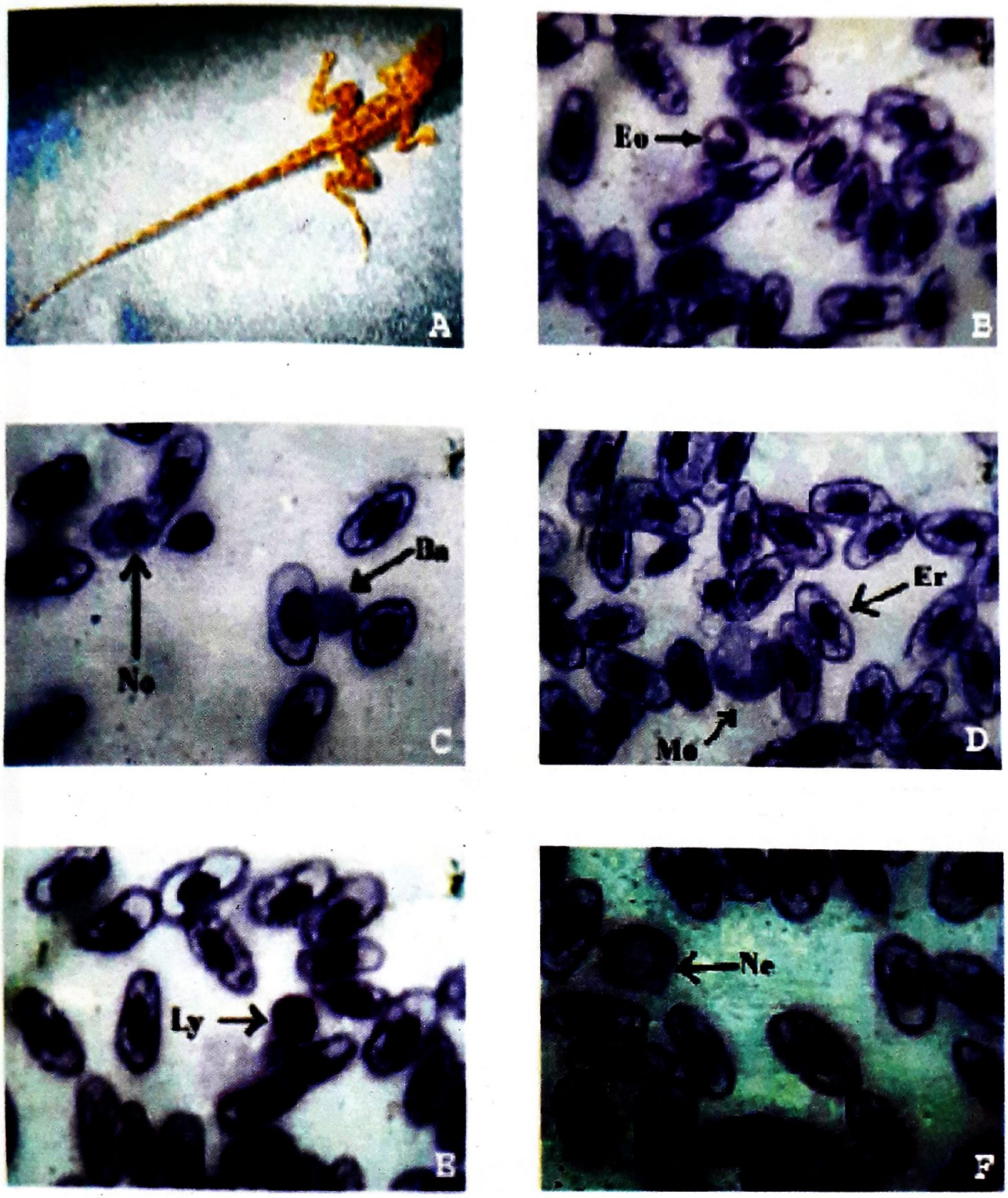
v. Neutrophils

These cells were circular with four to five lobes in the nucleus. The nucleus appeared dark while the cytoplasm was lighter in colour. The size of neutrophils ranged from 5.52 to 6.9 mm with an average diameter of 6.43 ± 0.79 mm in males. In females it ranged from 11.04 to 15.18 mm with an average diameter of 12.87 ± 2.10mm.

C. Differential leucocytes count (DLC)

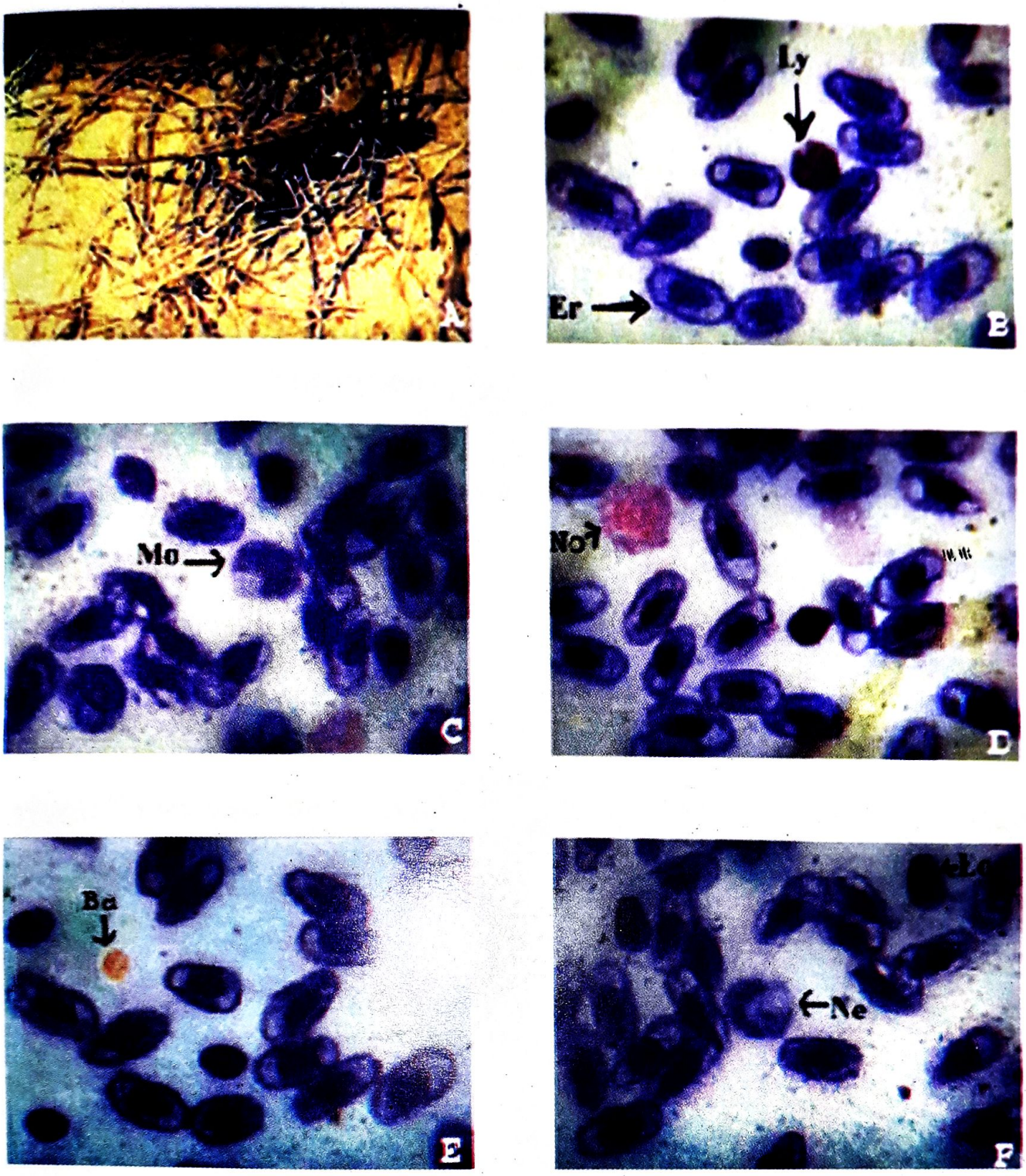
1. Lymphocytes: Total number of lymphocytes (small and large) varied from 32 to 37 % in males with a mean value of 34.66 ± 2.51 %. In females it ranged from 31 to 39 % (34.66 ± 4.04 %)

Figure 1.*Sitana ponticeriana* male and microphotograph of its different blood cells.



(Ly: Lymphocyte; Mo: Monocyte; Eo: Eosinophil; Ba: Basophil; Ne: Neutrophil; Er: Erythrocyte; No: Normocyte)

Figure 2. *Sitana ponticeriana* female and microphotograph of its different blood cells.



(Ly: Lymphocyte; Mo: Monocyte; Eo: Eosinophil; Ba: Basophil; Ne: Neutrophil; Er: Erythrocyte; No: Normocyte)

Table 1. Size and area of erythrocyte and its nucleus in *Sitana ponticeriana*

Sex	Size of cell			Size of Nucleus			Ratio S/S'
	Lin μm	Bin μm	Sin μm²	L'in μm	B'in μm	S'in μm²	
Male	(17.94 – 19.32)* 18.85 ± 0.79^	(11.04- 12.42) 11.73 ± 0.69	(155.5 –167.52) 163.44 ± 0.62	9.66	5.52	41.88	(3.71 – 4.0) 3.90± 0.1
Female	(20.07 – 22.08) 21.15 ± 0.79	(12.42 – 13.8) 13.33 ± 0.79	(195.7– 387.96) 221.42 ± 0.49	11.04	6.9	59.82	(3.27 – 6.48) 4.87 ± 1.60

L: Length B: Breadth S: Surface area L': Length B': Breadth S': Surface area

* - Range, ^ - mean ± standard deviation

Table 2. Size of different types of blood cells in *Sitana ponticeriana*

Cell type	Large Lymphocyte	Small Lymphocyte	Monocyte	Eosinophil	Basophil	Neutrophil
MaleSize in μm	(13.8 – 16.56)* 15.18 ± 1.38^	(8.28 – 11.04) 9.66 ± 1.38	(13.8 – 17.94) 15.63 ± 2.16	(8.28 – 11.09) 9.66 ± 1.38	(8.28 – 9.66) 8.73 ± 0.79	(5.52 – 6.9) 6.43 ± 0.79
Female Size in μm	(15.18 – 19.32) 17.47 ± 2.10	(8.28 – 12.42) 10.11 ± 2.10	(13.8 – 16.56) 15.18 ± 1.38	(11.04 – 3.08) 12.42 ± 1.38	(4.14 – 5.52) 4.59 ± 0.79	(11.04 – 15.18) 12.87 ± 2.10

* - Range, ^ - mean ± standard deviation

Table 3. Differential count of leucocytes (in%) in *Sitana ponticeriana*

Sex	Male					Female				
Cell Type	Lympho cyte	Mono cyte	Eosino phil	Baso phil	Neutro phil	Lympho cyte	Mono cyte	Eosino phil	Baso phil	Neutro phil
Percentage	(32 – 37)* 34.66 ± 2.51^	(2 – 8) 5 ± 3	(15 – 22) 18.6± 3.51	(3 – 8) 5.33± 2.51	(41 – 46) 43.3± 2.51	(31 – 39) 34.66 ± 4.04	(8 – 13) 10.6± 2.51	(10 – 20) 15.3± 5.03	(4 – 8) 6.0 ± 2	(37 – 48) 42.6±5.50

* - range, ^ - mean ± standard deviation

Table 4. Total number of RBC, WBC, and Haemoglobin content in *Sitana ponticeriana.*

Sex	Number RBC(mm^3)	Number of WBC mm^3	Haemoglobin in g/100ml
Male	(820000 – 840000)* 830000 ± 10000^	(14400 – 14800) 14600 ± 200	5.8
Female	(620000 – 660000) 640000 ± 20000	(11800 – 12200) 12000 ± 200	5.2

*- range, ^- mean ± standard deviation

Table 5. Amount of protein and cholesterol in the blood serum in *Sitana ponticeriana.*

Sex	Protein g/ l	Cholesterol g / l
Male	(47.76 – 70.16)* 59.698 ± 9.826^	(1.946 – 3.866) 2.665 ± 1.2
Female	(73.68 – 85.92) 79.826 ± 6.12	(7.982 – 10.586) 9.678 ± 1.546

*- range, ^- mean ± standard deviation

2. Monocytes: The percentage of monocytes ranged from 2 to 8 with a mean value 5 ± 3 in males. In females it ranged from 8 to13 with a mean value 10.66 ± 2.51.
3. Eosinophils: The percentage of eosinophils ranged from 15 to 22 with a mean value of 18.66 ± 3.51 in males. In females it ranged from 10 to 20 and the mean value was 15.33 ± 5.03.
4. Basophils: The percentage of basophils ranged from 3 to 8 with a mean value of 5.33 ± 2.51 in males. In females it ranged from 4 to8 with a mean value of 6 ± 2.
5. Neutrophils: The percentage of neutrophils ranged from 41 to 46 with a mean value of 43.33 ± 2.51 in males. In females it ranged from 37 to 48 with a mean value of 42.66 ± 5.50.

D. Total blood cell count (Table 4)

Total number of RBC ranged from 820000 to 840000/mm^3 (830000 ± 100000/mm^3) in males. In females it ranged from 620000 to 660000 /mm^3 (640000 ± 20000 /mm^3). Total number of WBC ranged from 14400 to 14800/mm^3 with a mean value of 14600 ± 200/mm^3 in males. The number ranged from 11800 to 12300/mm^3 with a mean value of 12000 ± 251.66 /mm^3 in females.

E. Hemoglobin (Table 4)

Hemoglobin content was 5.8 g/100ml of blood in males. In females it was 5.2 g/100ml of blood.

F. Blood serum (Table 5)

Serum protein: Protein content ranged vary from 47.76 to 70.16 g/l of blood serum with a mean value of 59.698 ± 9.82 g/l of blood serum in males. In females it ranged from 73.68 to 85.92 g/l of blood serum with a mean value of 79.826 ± 6.126 g/l of blood.

Serum cholesterol: Cholesterol content ranged from 1.946 to 3.866 g/l of blood serum with a mean value of 2.665 ± 1.2 g /l of blood serum in males. In females it ranged from 7.892 to 10.586 g/l of blood serum with a mean value of 9.678 ± 1.546 g/l of blood serum.

DISCUSSION

The length and breadth of RBCs were more in females than males. However, the female showed larger nucleus than males. The area of erythrocytes was also more in females than males. Among the WBCs, basophils were smaller in females than males. Haemoglobin content of females was 1.5 times less than males. Similar difference in the content of haemoglobin has been observed in the rainbow lizard *Agama agama* (Sodeinde and Ogunjobi, 1994). Total RBC and WBC count was 1.29 and 1.28 times more in males than respective females. However, the protein and cholesterol content of females was 1.33 and 3.63 times more than males. High value of cholesterol has been related to some degree of stress (Guillette et al., 1995). High concentration of cholesterol has also been associated with vitellogenesis (Dickinson et al., 2002).

CONCLUSION

The present study concluded that the hematological and biochemical parameters might change due to sex, inter species variation, environmental and ecological factors in poikilotherms in addition to activeness and inactiveness due to physiological stress. Further research on hematological values and biochemical values can be done to know more about the physiology of different type of reptiles.

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CONSUMPTION AND UTILIZATION OF DIET BY THE LARVAE OF ERI SILKWORM, *SAMIA CYNTHIA RICINI* BOISD. (LEPIDOPTERA: SATURNIIDAE)

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ABSTRACT

Larvae of Eri silkworm, *Samia cynthia ricini* Boisd were reared on four experimental diets i.e CC, CP, CA and CCr (Castor, Papaya, Ailanthus and Croton) inside the laboratory at a temperature of 23 ± 2° C and 60 ± 1% RH. Gravimetric method was followed for the estimation of food consumption, faeces voided and weight gained. Five nutritional parameters like Consumption Index (CI), Growth Rate (GR), Approximate Digestibility (AD), Efficiency of Conversion of Ingested Food (ECI) and Efficiency of Conversion of Digested Food (ECD) were studied. The values of CI, GR were increased with the advancement of larval stages in all the experimental diets. It was highest on CC and lowest on CCr leaves and the gradation of nutrition was concluded as CC>CA>CP>CCr.

Keywords: Food consumption, Eri Silkworm, nutrition.

INTRODUCTION

Feeding is an active and dynamic process during the larval development of holometabolous insects. The quantity, rate and food consumed by an insect has a great bearing on its survival, growth, developmental duration and final body weight. The quantity of food consumed directly or indirectly influences digestibility and conversion efficiency (Ramadevi et al., 1993). As food is of ecological importance, knowledge on types of food consumed and assimilation of food will enable us to understand nutritional physiology of the insect species. Insects have the ability to discriminate types of food and therefore they prefer to feed on the host plants,

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which provide them necessary nutrients in required concentration (Sanjayan and Ananthakrishnan, 1987).

MATERIALS AND METHODS

The cocoons of Eri Silkworm were brought from Eri seed station, Khurda and were kept in the laboratory. After the emergence of silk moth, they were allowed to mate and the eggs were collected. After 7-8 days when eggs hatched, they were brushed and kept in separate trays.

Forty replicates each consisting of ten larvae was reared separately. The larvae were weighed and known quantity of food was given to the larvae. Every day the weight of each replicate of larvae was taken and then the left over leaves were also weighed. The faecal matter was collected and weighed. In this way up to second instar, the larvae were grown on tender castor leaves. During this period temperature of the laboratory was maintained at 23 ± 2° C and 60 ± 1% RH.

After the second moulting i.e from the third instar stage, ten replicates of larvae were allowed to grow on the leaves of Ailanthus (CA); ten on Papaya (CP) and ten on Croton leaves (CCr). The remaining ten replicates were grown on castor leaves (CC).

Consumption and nutritional indices used in this study were calculated according to Sanjayan and Ananthakrishnan, 1987. All the parameters were calculated on dry weight basis using the following formulae:

Consumption Index (CI) = Weight of food eaten through one instar

Approximate Digestibility (AD) = $\frac{\text{Weight of food ingested - Weight of faeces}}{\text{Weight of food ingested}} \times 100$

Efficiency of conversion = $\frac{\text{Weight gained by the larvae}}{\text{Weight of food digested (Wt. of food ingested – Wt. of faeces voided)}}$

Gross Efficiency (GE) or Efficiency of Conversion of ingested food (ECI) =

$$\frac{\text{Weight gained by the larvae}}{\text{Weight of food ingested}} \times 100$$

Growth Rate (GR) = $\frac{\text{Net wt. gain per larva through one instar}}{\text{Duration of feeding days X Average weight of food taken per larva}}$

Assimilation (AS) = C - FU

Where C = total food consumed.

F = undigested food material.

U = nitrogenous excretory material.

RESULTS

Food consumption:

The average food consumption/larva/day was lowest (0.114gms) in 1st instar and highest (35.42 gms) in Vth instar larva fed on castor whereas on Ailanthus leaves it showed 22.94 gms, on Papaya, 20.55 gms and on Croton 16.12 gms. So, food consumption was highest in castor and lowest in Croton.

Egestion:

It was the maximum in the Vth instar larvae fed with castor i.e 5.55 gms and minimum with that fed with Croton i.e 3.90 gms.

Assimilation:

The average amount of assimilation / larva / day was highest (29.87gms) in the Vth instar larvae fed on castor leaves, whereas minimum (12.22 gms) fed on Croton leaves.

Metabolized food:

The metabolized food per larva per day during Vth instar was also highest (21.55gms) on castor and lowest (7.78 gms) on croton leaves.

Consumption indices:

The GR was highest in case of castor fed larva (0.527) and lowest in croton fed (0.206). The consumption of the approximate digestibility (AD) was also highest in Ist instar fed on CC (98.24) and lowest in IIIrd instar larvae fed with CCr (66.66).

Efficiency of conversion of ingested food (ECI) into body substance was highest in V^{th} instar larvae fed on CA leaves (12.65) and lowest in I^{st} instar larvae fed with CC leaves (2.70), whereas the efficiency of conversion of digested food (ECD) into body substances was highest (22.12) in III^{rd} instar larvae fed on CC leaves and lowest (4.14) in Ist instar fed on the same leaves. Rate of food intake revealed that the consumption index (CI) was highest in the V^{th} instar larvae fed on castor leaves (2.32) and lowest (0.144) in the II^{nd} instar larvae fed on the same leaves.

Table 1. Nutritional and growth parameters by Eri Silkworm larvae.

Instars	CI	GR	AD	ECI	ECD
			Castor leaf eater		
III	*1.65±0.02	*0.169 ±0.0013	*887.77±0.085	*12.193±0.013	*822.125±0.022
IV	*1.77±0.018	*0.42±0.0017	*86.13±0.088	*10.34±0.023	*13.28±0.019
V	*2.32±0.019	*0.527±0.0018	*84.33±1.087	*10.03 ±0.019	*12.09±0.018
			Ailanthus leaf eater		
III	*0.75±1.02	*0.096±0.0015	*70.45±0.01	*6.25±0.014	*10.15±0.016
IV	**1.25±0.0019	*1.13±0.0018	*76±0.011	** 10.93±0.019	*12.08 ±0.018
V	**1.85±0.0018	**2.36±0.0016	*80.62±1.012	*12.65±1.001	**12.95±0.002
			Papaya leaf eater		
III	*0.68±0.01	*0.085±0.0015	*68.9±0.01	*5.65±0.014	*9.25±0.016
IV	*1.15±0.016	*0.103±0.021	**74.6±0.013	**9.23±0.013	*11.95±0.016
V	*1.78±0.018	*0.216±0.025	**79.95±0.01	**12.05±0.012	*11.09±0.012
			Croton leaf eater		
III	*0.58±0.01	*0.08±0.002	**66.66±0.005	*4.27±0.002	*8.06±0.06
IV	*1.1±0.018	*0.101±0.021	**71± 0.012	**10.23±0.003	**12.15±0.004
V	*1.55±0.012	*0.206±0.009	**75.8±0.013	**11.05±0.018	**13.091±0.001

*Significant at 1%level($p<.001$)

**Significant at 5% level ($p<.005$)

DISCUSSION AND CONCLUSION

During the present study it was seen that the maximum food was consumed during the 5th instar. It confirmed the earlier reports that the great mass of food was ingested during the last or last two instars of the lepidopterans. It has been reported that *Bombyx mori* consume about 97% food during two instars and about 99% during the last three instars (Waldbauer, 1964). The present results also coincides with the result of Maribashetty et al., (1999) where food consumption was higher during IVth instar.

In insects the feeding is governed by the quantity of food, water content and physicochemical characters of food material. In the present study, the consumption index (CI) was lowest during first instar and highest during last instar in the larvae growing on castor leaves. In other dietary regimes such as CP, CA and CCr the consumption index (CI) was lowest during the IIIrd instar stage and gradually increased with the advancement of larval stages. But, this result does not coincide with the result of Mohanty and Mittra (1992) where CI was highest for Ist instar and lowest for Vth instar. The growth rate (GR) in this experiment increased with the advance ment of larval stages and is maximum in Vth instar which is also opposite to that of Mohanty and Mittra (1992).

The approximate digestibility (A.D.) decreased as the larvae grew old , as has been reported is other lepidopteran larvae such as *Danaus chrysippus* (Mathavan and Bhaskaran, 1975) and *Pericallia ricini* (Chockalingam, 1979). It confirmed the fact that the efficiency utilization is likely to differ from stage to stage and from instar to instar (Waldbauer, 1964). Similar results have been reported by many earlier workers (Hiratsuka, 1920; Scriber and Slansky, 1981; Latheef and Harcourt, 1972; Kogan and Cope, 1974). But in other secretory host plants such as CA, CP and CCR, the A.D. gradually increases from third to fifth instar. It may be due to the fact that the young larvae were more selective feeders and chose more digestive foliage from the intervein portions of the leaf than the older larvae which fed more indiscriminately (Bailey, 1976).

While the A.D. of the larvae decreased with increasing age, the ECI and ECD showed an increasing trend. However, both the ECI and ECD decreased in the Vth instar in all experimental dietary regimens. In corroboration Scriber and Slansky (1981) reviewed that the ECI may increase, decrease or show little change. These changes are influenced by the digestibility of food, its nutritional values and the level of nutrient intake (Waldbauer, 1964). The gradual increase in CCI during the first four instars corroborated the finding of Latheef and Harcourt (1972) and Kogan and Cope (1974).

From the present study, on four experimental diet i.e. CC, CA, CP and CCr, it would be concluded that there is gradation of diets taking all the nutritional and growth parameters along with energy budget. Performance on Castor (CC), Papaya (CP), Croton (CCr) and Ailanthus (CA) is in the grade CC>CA>CP>CCr.

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RESTORATION, CLEANING AND PROCESSING OF AFRICAN BLACK RHINO (*DICEROS BICORNIS*), SKELETON EXHIBIT IN REGIONAL MUSEUM OF NATURAL HISTORY, BHUBANESWAR, ORISSA, INDIA

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ABSTRACT

The mummified skeleton remains of a Black Rhinoceros *Diceros bicornis*, which was possession of Van Ingen of Mysore since the death of the animal has been procesed. The specimen was donated to Regional Museum of Natural History, Mysore in December 1998. Recently the specimen was transferred from RMNH, Mysore to RMNH, Bhubaneswar. Processing and mounting of the skeleton of a Black Rhinocerus, *Diceros bicornis* reported herein with a detailed characteristic descriptions and morphometric measurement. The profile length of the mounted skeletons is 337.5 cm and height is 142.5 cm.The restored whole skeleton of Black rhinoceros will not only be used for educational exhibition but for study of comparative osteology of different extinct and living rhinoceros of the world.

Key words: Black Rhinoceros skeleton, articulation, display, education, mounting.

INTRODUCTION

Black rhinoceros, *Diceros bicornis* may be called as great giant living genus of land mammals except for *Elephas, Loxodanta*, *Ceratotherium* and perhaps *Hippo*. It is classified as endangered by IUCN & CITES.

We report here the processing and mounting of a skeleton of African Black Rhinoceros *Diceros bicornis* with an attempt to record and discuss the relevant experiences and

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problems. The mummified skeleton remains of the Black rhinoceros was possession of Van Ingen, a taxidermist of Mysore since the death of the animal in 1984. The specimen was donated to Regional Museum of Natural History in 1998. It was transferred from RMNH, Mysore to RMNH, Bhubaneswar. The specimen of the Black Rhinoceros was examined with detailed character analysis and morphological measurements. The present report is made on the skeletal morphometric data of the living rhinoceros sample for use in future studies. The specimen is displayed in the Regional Museum of Natural History, Bhubaneswar.

MATERIALS AND METHODS

The material in this report is the skeleton remains of a male Black rhinoceros, *Diceros bicornis*. It lived in Mysore zoo until it died on 4th February1984. The record for its age from Mysore zoo is not available. The whole mummified skeleton with flesh was brought to RMNH, Mysore in 1998 and ultimately the specimen was brought to RMNH, Bhubaneswar (Fig.1). The skeleton was prepared after 21 years of its death.

The first step was to separate whole skeleton into disarticulated pieces. Flesh was removed from bones. Bones were then bleached and preserved for further restoration. All the skeletal parts were assembled systematically as it was in the initial stage of recovery. Wooden framework was necessary to hold such a huge skeleton during assembling.

In the final step morphologic character of the Black rhinoceros was investigated by detailed craniometrical and osteometrical studies. These measurements were taken with calipers (straight line measurement) and with a flexible tape for curved measurement (Von-den Driesh, 1976).

A. Cleaning of Skeletal parts

The whole mummified body was subjected to softening in 1% KOH solution. After softening of the flesh each part of the skeleton was carefully disarticulated. The bones were carefully numbered from first vertebra (Atlas) to the tip of the tail (Caudal) to keep whole skeletal system in order as per natural arrangement of bones. Bones were cleaned to remove the flesh till the bones were free from any cartilage or flesh. A detergent (Surf) was used to reduce the fatty substance from bones. The clear bones were dipped into 1% H_2O_2 solution for one hour (Fig.2). After bleaching, the bones were dried with the

help of dryer. Care was taken to make the bone moisture free to prevent fungal and insect infestation (Fig.3)

B. Assembling of skeletal parts:

Initially the skull was properly articulated and locked to keep lower jaw fixed with the skull. As per the diameter of the neural canal of the caudal bone, galvanized iron wire (2mm) comprising the length of the whole body was first inserted and subsequently the number of wires increased proportionally with the increase of the neural canal diameter to fix the vertebral column as per the spinal column position. Appendicular skeletons comprising femur, tibio-tarsus, meta-tarsus and phalanges were arranged as per the original drawing taken before dismantling the location of each bone. Each part of appendicular skeleton was fixed with the help of steel and brass screw as per the width and weight of the bones. The position of the screw was meticulously placed to avoid visibility at a glance. The pair of hind limbs were thus assembled and kept for further joining with the pelvic girdle. Likewise pair of fore legs was also assembled meticulously giving a moving posture to the leg. The ribs were fixed on the requisite vertebra. Ribs attached with both the processes were placed on the lateral side of the each vertebra (T1 to T20). The remains of delicate sternal cartilage were precisely cleaned and re-assembled as per initial arrangement. It was really difficult to place forelegs as they were mostly floating. Both the legs were tied with galvanized iron wire and put on rest on available space to the top of second vertebral crest on the anterior site of thoracic region. The placement of scapula was carefully done on the vertebral column keeping the required space with the help of 10 mm purpex sheet moulded to conceal the visibility from outside in between inner side of scapula and the lateral side of neural spine of thoracic region (i.e. T2). All the ribs were systematically arranged with the help of coiled aluminium wire so as to give a proper shape to the vertebral ribs (Fig.4). The anterior portion of first vertebra (atlas) was fixed with foramen magnum of the skull after inserting the bunch of wire, which was the backbone of the whole vertebral column. Here an unique system was evolved to keep the whole skeleton (weighing about 90 Kg) in a floating position with the help of strong steel control cable with jam bolt on concealed iron bar which can stand on the weight of the whole skeleton. Finally the skeleton was displayed in a teller mate showcase with fixed 6-sided glasses to have a complete view of the skeleton. Measurement of important skeletal parts was done following cleaning (table 1- 17).

RESULTS AND DISCUSSION

Total number of bones recovered except skull and mandible was 186. The rib bones were comparatively longer in size. The sesmoid bone of foot was the smallest bone in the present study because the bones of ear ossicles were missing in the specimen. All the bones were measured following cleaning. Details of measurement are given in table 1 to 17.

Since, the carcass of the African black rhinoceros was mummified with flesh, recovery was difficult. The carcass was manually dissected and cleaned. The chemical cleaning method of Taylor (1967) was followed for bleaching of bones. Permanent mounting of skeletal bones often limits the accessibility of the specimen for scientific study. In view of future study available, the removable arrangement of bones and external armature mounting with possible dismantling was done. In the standing posture, the highest point in the black rhinoceros skeleton was the tip of the sagittal crest of the skull. This was confirmed by observing a few live domestic rhinoceroses in zoo standing in a normal position. However, in some museum specimens (e.g., the American Museum of Natural History), the skull is placed slightly degraded and the height of the rhinoceros is measured at the tip of the 2nd thoracic vertebra. Whereas, the shoulder height of the rhinoceros is considered as the standard for the height of a rhinoceroses, in a situation where the skull or 2nd thoracic vertebra is higher than natural on a mounted skeleton, the shoulder height can be obtained by reaching the tip of the scapulae. The shoulder height of the black rhinoceros is 142.5 cm and the height at the tip of skull is 183.7 cm.

From the study of skeletons it was concluded that the specimen was a mature male and its length and height are 340 cm and 143 cm, respectively, within the ranges of Foster's (Foster, 1966) measurements. The potential longevity of black rhinoceros is 40-50 years and males may mature in 3 years (Groves, 1972). Since mammals have approximately limited growth in skeleton until sexual maturity, the specimen we obtained would be taken as a representative individual of sexual maturity. Its skull and bones are available as criteria for further comparative study in anatomy with other fossil remains of extinct species of the Rhinocerotidae.

Table 1. Measurements of the skull of *Diceros bicornis* (in mm)

Condylobasal length	576
Interorbital width	313
Maximum palate length	97
Minimum palate width	35
Nasal length	216
Orbital Diameter	91
Overall length	614
Palate length	226
Postorbital constriction	71
Zygomatic width	38.7
Greatest breadth of skull	297
Greatest palatal breadth	249

Table 2. Measurements of the mandible of *Diceros bicornis* (in mm) Mandible Left side.

Length from the angle	464
Length from the condyle	488
Length of the horizontal ramus	263
Length of the diastema	46
Height of the mandible	86

Table 3. Measurements of the cheek teeth of *Diceros bicornis* (in mm)

	Right		Left	
	Length	Breadth	Length	Breadth
Upper maxillary teeth				
P1	33	31	35	33
P2	44	42	45	43
P3	46	56	52	55
M1	32	61	33	62
M2	37	68	36	69
M3	72	59	71	54
Lower dentary teeth				
P1	36	21	41	23
P2	38	39	39	42
P3	37	47	39	31
M1	31	53	34	56
M2	38	31	41	32
M3	55	34	58	39

P: premolar; M, molar

Table 4. Measurements of scapula of *Diceros bicornis* (in mm)

Scapula	Right	Left
Height	508	506
Diagonal height	450	459
Greatest dorsal length	271	279
Length of the glenoid cavity	113	101
Breadth of the glenoid cavity	100	95

Table 5. Measurements of pelvis of *Diceros bicornis* (in mm)

Greatest length of one half	1579
Length of the acetabulum including the hip	107
Length of the acetabulum on the rim	99
Length of the symphysis	229
Smallest height of the shaft of ilium	98
Smallest breadth of the shaft of ilium	62
Inner length of the foramen obturatum	102
Greatest breadth across the tubera coxarum	1867
Greatest breadth across the acetabula	430
Smallest breadth across the bodies of the ischia	239

Table 6. Measurements of humerus of *Diceros bicornis* (in mm)

Humerus	Right	Left
Greatest length of the lateral part	456	455
Greatest length from caput	375	373
Greatest breadth of the proximal end	113	115
Depth of the proximal end	183	186
Smallest breadth of diaphysis	77	78
Greatest breadth of the distal end	166	168
Greatest breadth of the trochlea	120	123

Table 7. Measurements of radius of *Diceros bicornis* (in mm)

Radius	Right	Left
Greatest length	365	364
Length of the lateral part	335	332
Greatest breadth of the proximal end	125	122
Greatest breadth of the facies articularis proximalis	108	111
Greatest breadth of the distal end	118	117
Greatest breadth of the facies articularis distalis	91	89

Table 8. Measurements of ulna of *Diceros bicornis* (in mm)

Ulna	Right	Left
Greatest length	485	479
Length of the olecranon	158	150
Greatest breadth across the coronoids process	114	119

Table 9. Measurements of metacarpus of *Diceros bicornis* (in mm)

Metacarpus	Right			Left		
	II	III	IV	II	III	IV
Greatest length	130	188	153	165	192	142
Greatest breadth of the proximal end	53	76	54	49	75	54
Smallest breadth of the diaphysis	40	54	41	39	56	41
Smallest circumference of the diaphysis	111	138	108	115	140	110
Greatest breadth of the distal end	63	78	58	52	79	61

Table 10. Measurements of femur bones of *Diceros bicornis* (in mm)

Femur	Right	Left
Greatest length	514	512
Greatest length from caput femoris	503	502
Greatest breadth of the proximal end	219	217
Smallest breadth of diaphysis	80	82
Smallest circumference of diaphysis	231	230
Greatest breadth of the distal end	145	143

Table 11. Measurements of fibula of *Diceros bicornis* (in mm)

Fibula	Right	Left
Greatest length	303	303

Table 12. Measurements of tibia of *Diceros bicornis* (in mm)

Tibia	Right	Left
Greatest length	372	371
Lateral length of the outer side	329	324
Greatest breadth of the proximal end	139	136
Smallest breadth of the diaphysis	64	65
Smallest circumference of the diaphysis	214	214
Greatest breadth of the distal end	105	102
Greatest depth of the distal end	86	83

Table 13. Measurements of patella of *Diceros bicornis* (in mm)

Patella	Right	Left
Greatest length	102	100
Greatest breadth	78	75

Table 14. Measurements of metatarsus of *Diceros bicornis* (in mm)

Metatarsus	Right			Left		
	II	III	IV	II	III	IV
Greatest length	134	168	157	146	161	139
Greatest breadth of the proximal end	43	59	39	29	61	46
Smallest breadth of the diaphysis	31	47	31	33	49	33
Smallest circumference of the diaphysis	98	127	91	94	125	97
Greatest breadth of the distal end	50	72	50	46	73	52

Table 15. Measurements of atlas of *Diceros bicornis* (in mm)

Greatest breadth over the wing	346
Greatest breadth of the facies articularis caranialis	153
Greatest breadth of the facies articularis chordalis	143
Greatest length from the facies articularis to the facies articularis chordalis	134
Length of the arcus dorsalis, melios	88
Height	166

Table 16. Measurements of axis of *Diceros bicornis* (in mm)

Greatest breadth across the processus articulares	68
Greatest breadth of the facies articularis	153
Smallest breadth of the vertebra	52
Greatest breadth of the facies terminalis	90
Greatest height	205

Table 17. Measurements of sacrum of *Diceros bicornis* (in mm)

Greatest length on the ventral side	210
Greatest breadth	203

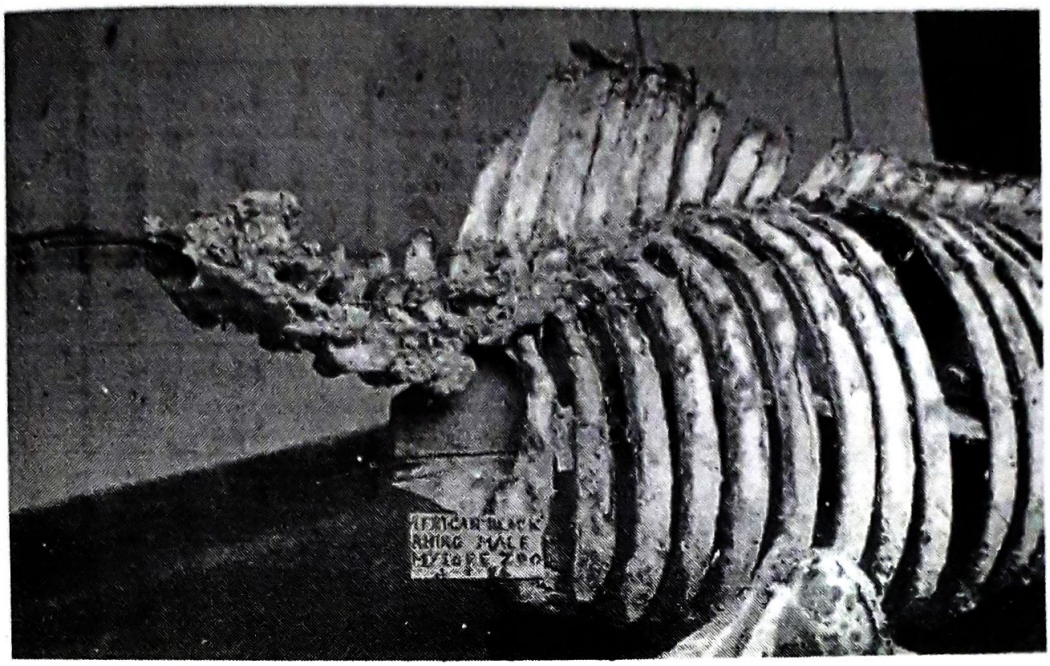

Fig.1 Mummified skeleton of *Diceros bicornis* at Regional Museum of Natural History, Bhubaneswar.

Fig.2. Disarticulated skeleton of *Diceros bicornis* treated with chemicals at Regional Museum of Natural History, Bhubaneswar.

Fig. 3 Disarticulated skeleton of *Diceros bicornis* after cleaning and bleaching at Regional Museum of Natural History, Bhubaneswar.

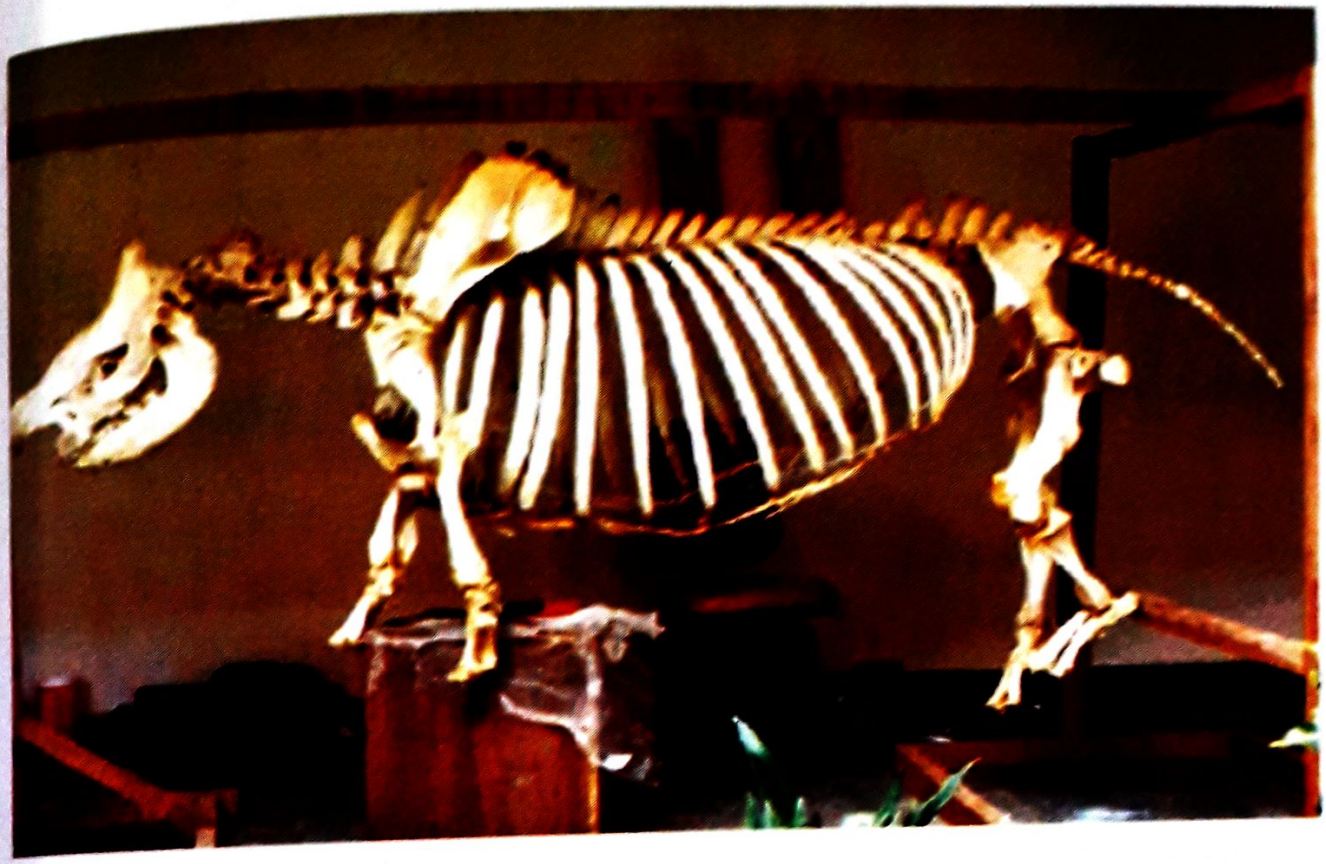

Fig.4 Restored whole skeleton of *Diceros bicornis* at Regional Museum of Natural History, Bhubaneswar.

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We would like to express our gratitude to Van Ingen of Mysore for donating the African black rhinoceros to the Regional Museum of Natural History, Mysore. Our special thanks to all members of Regional Museum of Natural History, Bhubaneswar for preparation of the African black rhinoceros skeleton as a specimen of collection of the museum. We also thank very much the anonymous reviewers for helping us to improve this manuscript.

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ORGANIZATION OF THE DEVELOPING BRAIN IN THE TADPOLE OF *POLYPEDATES MACULATUS*

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ABSTRACT

Using conventional histological techniques the organization and cytoarchitecture of the brain of tadpoles of the common Indian tree frog, *Polypedates maculatus* was studied. The brain of tadpoles showed distinctly demarcated telencephalon, diencephalon, mesencephalon and rhombencephalon with their corresponding cavities. The telencephalon had a rich population of neurons, where the cell bodies were mostly concentrated in the periventricular area and the fibers ran along the outer margin. The diencephalon was distinguished by the presence of the third ventricle, which was surrounded by a dense population neuronal perikarya. The mesencephalon which serves as the major relay station in the brain was marked by the appearance of certain important mesencephalic regions like optic lobe and tectum. The caudal most portion of the brain, the rhombencephalon, showed its characteristic rhombocoel and the typical pattern of arrangement of cells and fibers. Significance of the development and differentiation of the brain and the involvement of neural circuits in the integration of various functions in the tadpole brain have been discussed.

Key words: Brain, *Polypedates maculatus*, Tadpole

INTRODUCTION

The complex life cycle of amphibians, particularly the anurans, requires that the larvae and adults occupy different ecological stations. Thus the interactions between these two stages of amphibian life cycle provide a unique platform for the ontogenetic as well as phylogenetic studies among tetrapods. Small size, relatively short life span, absence of

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sexual behaviour, low trophic position, abundant availability and easy maintenance of tadpoles make them good subjects to study biological processes and their interactions. The combined life cycle and considerable developmental plasticity in amphibia provide the investigator an opportunity to address issues relating to environmental, populational and biological influences on morphology, growth, metabolism, larval duration and metamorphosis in tadpoles. No doubt studies on anuran tadpoles have made significant contribution in many areas of biological investigation, which have been useful in defining general principles and the nature of interactions operating at the individual, population and community levels. But most of the studies on anuran larval biology embrace taxonomic and morphologic schemes. These are, for example, studies on character variation and ecology (Duellman, 1978), functional and evolutionary morphology (Wassersug, 1980), swimming (Wassersug and Hoff, 1985), feeding ecology (Lannoo et al., 1987) and biodiversity (Maeda and Matsui, 1989; Chou and Lin, 1997) of tadpoles. But the neurobiology of anuran tadpoles is less known than that of the adults. During the transition from the aquatic to terrestrial life, the nervous system is remodelled to integrate the inputs from two different habitats. Such a condition in life necessitates certain modifications in the structure and function of the tadpole brain.

Studies on relatively simple brain of tadpoles have provided some insights into the development of certain neural components during embryonic life. For example, growth and development of cerebellum (Hauser et al., 1986); formation of spinal cord (Nordlander 1986), and sexual dimorphism of the brain (Gorlick and Kelley 1987) have been studied in tadpoles. Notwithstanding these reports, the organisational and cytoarchitectural details of the brain is not known in tadpoles as it is known for adult amphibians. Therefore, the organization and topography of neurons in the major brain regions, such as telencephalon, diencephalon, mesencephalon and rhombencephalon have been described in the brain of tadpoles of the common Indian tree frog, *Polypedates maculatus* using conventional histological techniques.

MATERIALS AND METHODS

Egg clutches of *Polypedates maculatus* were collected from the local breeding ground, transported to the laboratory and reared under normal environmental conditions until

metamorphosis. The embryo/ larvae were kept in bowls and fed with boiled spinach leaves and boiled egg yolk. The bowls were cleaned frequently to remove the fecal matter and to prevent bacterial growth. The staging of the larvae was done following the Gosner table (Gosnar, 1960).

Six larvae each at an interval of 5 stages from stage 20 onwards were given an overdose of anaesthesia and fixed in sublimate formol (saturated aqueous solution of mercuric chloride 9 parts: formaldehyde 1 part) for 24 hours. Fixation followed a thorough washing in running tap water and the materials were dehydrated in ethanol series, cleared in xylene, embedded in paraffin wax and sectioned at 10µm thickness serially in transverse and sagittal planes. Then the sections were dewaxed in xylene and stained with haematoxylin – eosin to study the anatomy, and organization of nerve cells in the tadpole brain. The key nuclei and regions of the brain were identified by matching the plane with the anuran tadpole brain described by Lannoo (1999).

RESULTS

The brain of the tadpole of the Indian tree frog showed three divisions: prosencephalon, mesencephalon and rhombencephalon. The anterior most division, the prosencephalon further contained two regions, i.e., telencephalon and diencephalon. The telencephalon contained its original cavity, the telocoel (Fig.1). Initial differentiation of cerebral hemispheres occured at around stage 20-22 , but the two cerebral hemispheres only became large and distinct during the later stages of development. From around premetamorphic stage 30, the compression of lateral ventricles enlarged and the constriction of two telencephalic vesicles became more prominent. Thereafter the cerebral hemispheres continued to develop, differentiate, became thick and enlarged with concomitant reduction in the size of ventricles till the prometamorpic stage 40 (Fig.2). Most of the telencephalic areas had a rich population of neuronal cells. The cell population, however, was concentrated in the periventricular area while the fibers ran along the outer margin of the telencephalon (Fig.1). The diencephalon, the other division of prosencephalon, was distinguished by the presence of the third ventricle, which extended from the caudal telencephalon to diencephalon. But the third ventricular cavity was large in the diencephalic region, which is known as diocoel (Fig.3). The third ventricular cavity extends in several directions, i.e., laterally into optic vesicles, anterodorsally beneath the

epiphysis, anteroventrally into optic recess and posteroventrally into infundibulum. The diencephalon showed a dense population of cells surrounding the third ventricle (Fig.3).

The area of the brain that lie between the rostral prosencephalon and caudal rhombencephalon is mesencephalon which contains the major pathways of nerve tracts. Although the initial differentiation of mesencephalon occurred at around stage 21-22 in the embryonic brain of *Polypedates maculatus*, a fairly well developed and well.

FIGURE LEGENDS

Fig.1. Sagittal section of the tadpole brain at stage 35 showing aggregation of cells surrounding the telecoel (Tc) in the telencephalon (Tel). x 70

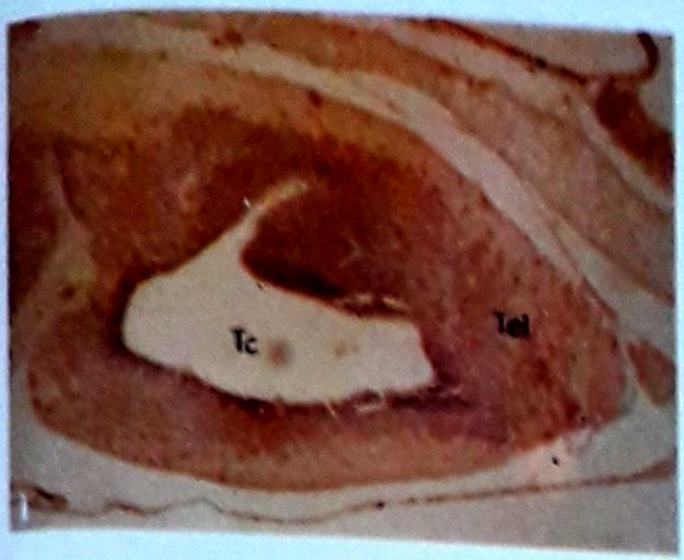
Fig.2. Transverse section of the telencephalic region at stage 40 of the tadpole brain. x 70

Fig.3. Transverse section of the tadpole brain at stage 40 in the diencephalic region (Dien). Note the dense population of neuronal cells surrounding the third ventricle (3v). x 70

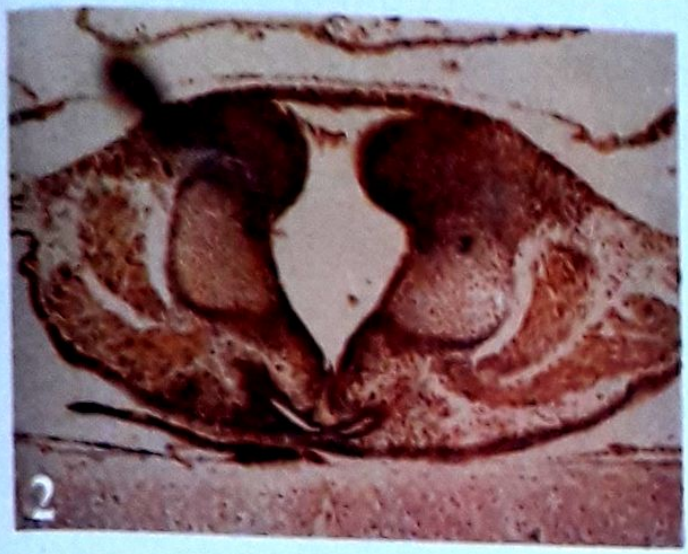
Fig. 4. Sagittal section of the tadpole brain at stage 35 showing mesencephalon (mes) and its regions optic lobe (OL), optic ventricle (OV) and tectum (Tc). x 70

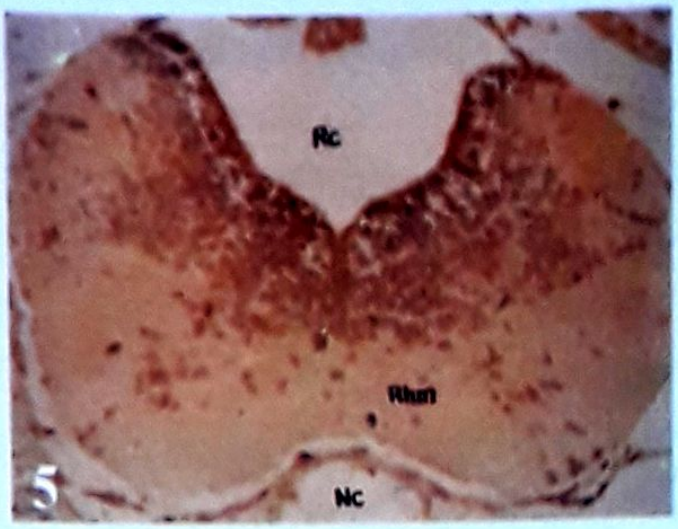
Fig.5. Transverse section at stage 25 of the embryonic brain showing rhombencephalon (Rhm) and rhombocoel (Rc). Notochord (Nc) is also seen in the picture. x 140

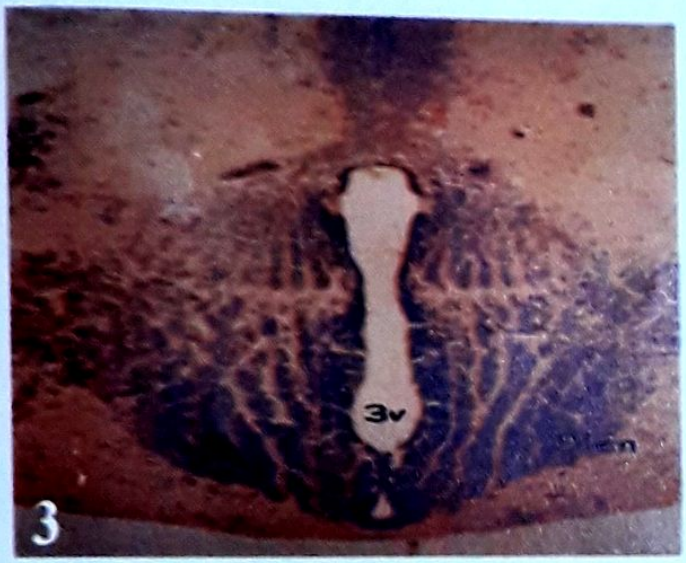
Fig.6. Sagittal section of the tadpole brain at stage 35 in the rhombencephalic area. x 70

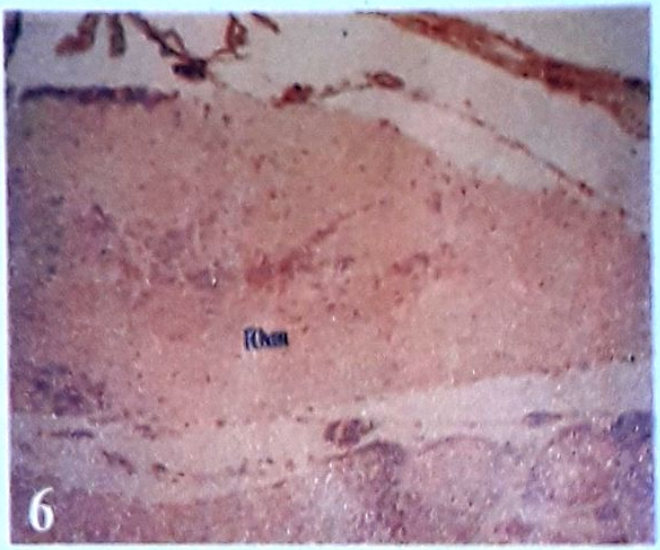

Tc
Tel
1


Tc
Ov
Mes
4


2


Rc
Rhm
Nc
5


3v
3


Rhm
6

differentiated midbrain did not occur until the premetamorphic stage 30-35 (Fig.4). At this stage (30-35) the mesencephalon showed its important regions like the optic lobe and tectum. A dense population of neuronal cells were observed in major mesencephalic areas, whereas the fibers were confined to their respective outer margins (Fig.4). The mesencephalic optic lobe had a clear optic ventricle and the tectum formed a thin roof over the optic ventricles (Fig.4). The cavity of the mesencephalon, the mesocoel, connects the rhombocoel with the third ventricle. The full development of the mesencephalon, however, did not occur until metamorphosis.

The caudal most portion of the brain, the rhombencephalon, was observed at about stage 20-25 in the larva of *Polypedates maculatus* (Fig.5). Anatomically rhombencephalon presented an elongated structure. It was wider at the rostral end and gradually tapered caudally (Fig.6). In the rhombencephalon the neuronal cells were situated close to the rhombocoel while the fibers ran laterally(Fig.5).

DISCUSSION

The amphibian development generally explains a fundamental pattern of organogenesis which is seen in other vertebrates too. Being pioneers among terrestrial vertebrates, amphibians are neither fully aquatic nor have they mastered the land. They are positioned between the two. During the early stages of development most amphibians experience an aquatic larval phase. But in the following shift from aquatic to terrestrial mode of life, the amphibian morphology and physiology undergo striking changes which help them adapt the new world. During this transformation many aspects of larval biology witness progressive changes. Of all such morphological and physiological transformations, the most complex and intricate mechanism is the remodelling of the neural circuitry during metamorphosis in amphibians.

In most anurans, the neural circuitry of the larva is remodelled at metamorphosis. During this remarkable transition, the nervous system graduates to cope with the shift and

integrates diverse informations. In any case, the neurobiology of anuran tadpoles is less known than that of the adults. Some studies, however, have explained the development and differentiation of brain areas in the amphibian embryo. For example, it has been shown that mesodermic induction of ectoderm to neuroectoderm occur during development in anuran embyos (Hamburger, 1988). Further, in the larval life, anatomical components like cerebellum (Hauser et al., 1986) and functional differentiation like sexual dimorphism (Gorlick and Kelley,1987) occur in the brain of a tadpole. In the present study in the tadpoles of *Polypedates maculatus*, it was found that although the major divisions of brain, i.e., telencephalon, diencephalon, mesencephalon and rhombencephalon were distinctly demarcated by stage 25 (11 mm in length), they continued to grow, develop and differentiate till metamorphosis.

In tadpoles it is known that the telencephalon integrates sensory inputs and initiates appropriate motor outputs with the involvement of the diencephalon (Northcutt, 1981). The telencephalon receives inputs from the olfactory bulbs and the terminal nerve (Hofmann and Meyer, 1989). The same authors have also reported that in *Bufo* and *Xenopus* the terminal nerve projects to the telencephalic septum and the olfactory nerve projects to the medial wall of the telencephalon. Another telencephalic area associated with the olfactory system is amygdala. Neurons of amygdala project to hypothalamus (Kemali and Guglielmotti, 1977). The posterior telencephalon contains pallial and striatal areas beside the lateral ventricles. The septum lies ventral to medial pallium and the amygdala lies posterior to striatum. Fibers from diencephalic thalamus and mesencephalon join the olfactory fibers and establish synaptic stations at telencephalic septum and striatum. Similarly the posterior brain regions are connected with the telencephalon with the medial and lateral forebrain bundles. The medial forebrain bundle establishes the link between olfactory centre of septum and hypothalamus. Specific projections from the septum enter ventral thalamus, preoptic area and anterior hypothalamus as well (Wilczynski and Northcutt, 1983a).

The striatum and amygdala also provide fiber inputs to the ventral thalamus and preoptic area through the lateral forebrain bundle (Wilczynski ad Northcutt 1983b). From the ongoing account it is evident that the septum, amygdala, pallium and striatum of the telencephalon are involved in the integration of olfactory information. Likewise several neural circuits concerned with the integration of various other functions in the tadpole brain develop from the premetamorphic through the prometamorphic changes to the metamorphic climax. Thus it is likely that the brain of a tadpole continually undergo changes in its structure and function which eventually equip the animal to cope with the postmetamorphic challenges of life.

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TISSUE SPECIFIC DIFFERENTIAL MODULATION OF ACTIVE OXYGEN METABOLISM IN MOUSE BY HEXACHLOROCYCLOHEXANE

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ABSTRACT

In the present study the effect of γ-hexachlorocyclohexane (20 mg/kg body weight/day for 30 days, intra-peritoneal) on oxidative stress parameters in various tissues of Swiss albino mouse was compared. In case of liver, no significant change in the level of lipid peroxidation, hydrogen peroxide content and catalase activity was observed in response to γ-hexachlorocyclohexane treatment. Although activity of superoxide dismutase, contents of water-soluble organic hydroperoxides and reduced glutathione were decreased in the hepatic tissue of the pesticide treated animals, a significant increase in the activities of glutathione peroxidase and glutathione S-transferase was noticed. Activities of superoxide dismutase and catalase of kidney did not change in response to the pesticide treatment, however, an augmentation in glutathione peroxidase activity with simultaneous decline in the levels of reduced glutathione, lipid peroxides and hydrogen peroxide were observed in the tissue. A significant decline in the contents of water-soluble organic hydroperoxides, lipid peroxides and the activities of superoxide dismutase and glutathione peroxidase was recorded in the cerebral cortex in response to the pesticide treatment which was accompanied by increase in hydrogen peroxide level and activities of catalase and glutathione S-transferase. The results of the present study surmise tissue specific modulation of antioxidant defences by γ-hexachlorocyclohexane.

Key words: Hexachlorocyclohexane, mice tissues, lipid peroxidation, antioxidant defence.

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INTRODUCTION

One of the oldest organochlorine insecticides still widely used for agricultural and medicinal purposes is 1, 2, 3, 4, 5, 6-Hexachlorocyclohexane (HCH) (Suwalsky et al., 2000; Walsh and Stacco, 2000; Walsh et al., 2000). Like other organochlorines, HCH enters into animal tissues either through food chain, respiration or dermal contact from the environment and is deposited in the adipose tissue as well as in the membrane bi-layer of all cells (Zhu et al., 1986; Lopez-Aparicio et al., 1988). Even though the use of HCH has been reduced and its production is banned in most of the countries including India, presence of the pesticide is reported in the environment (Pandit and Sahu, 2001). Recently, several reports indicated increased risk to humans by HCH as a consequence of its incorporation into the food chain (Kole et al., 2001; Senthilkumar et al., 2001).

Involvement of reactive oxygen species (ROS) is currently believed as one of the mechanisms through which xenobiotics exert their deleterious effects on animal tissues. A wealth of literature records a positive correlation between toxicity of organochlorine pesticides and oxidative stress as a mode of tissue injury due to their ability to participate in microsomal oxidation as oxygen acceptors generating ROS and to cause oxidative deterioration in membranes (Junqueira et al., 1988; Videla et al., 1990; Hassan et al., 1991; Bagchi and Stohs, 1993; Bagchi et al., 1993; Sahoo et al., 2000). Recently, Junge et al., (2001) demonstrated that lindane (γ-HCH) sensitizes the liver to the damaging effects of iron overload by providing an added enhancement to the oxidative stress status in the tissue, which in turn might be contributing to the alteration of the respiratory activity of Kupffer cells and the development of an inflammatory response.

Brain, liver and kidney are metabolically active organs. Nevertheless they differ from each other for their respective physiological functions. Although the general metabolic pattern is quite similar among them, a subtle difference regarding their biochemical composition and metabolism is well documented. For example, while brain is considered highly vulnerable to oxidative stress due to its poor antioxidant defenses and high PUFA content, the liver is not only metabolically very active but also considered as one of the tissues having high rate of reactive oxygen species production (Halliwell and Gutteridge, 2001). While most of the xenibiotics are metabolically processed in liver, kidney is responsible for the excretion of metabolites (Engst et al., 1977). Comparison of results of several studies in past have clearly

indicated that responses of antioxidant enzymes and extent of oxidative damages inflict to tissues by HCH not only differ from one tissue to another but also from one species to another (Munir et al., 1984; Arisi et al., 1994; Puri and Kohli, 1995; Samanta and Chainy, 1997; Samanta et al., 1999; Sahoo et al., 2000). The studies were mostly confined to rats. Not much information is available on mice. And whatever information is available they are mostly confined to one tissue at a time and no attempts were made to compare the effects of HCH on different organs with respect to same dose and duration of the pesticide. Recently we have shown that response of testis of mice to HCH is quite different than rat (Samanta and Chainy, 2002). It is believed that comparison of responses of antioxidant enzymes and extent of oxidative damages occurred in tissues by the same dose and duration of HCH treatment can offer clues to understanding the physiological basis of different tissue responsiveness to HCH. Therefore, in the present study levels of antioxidant enzymes, oxidative stress parameters and reduced glutathione content were measured comprehensively in brain, liver and kidney of mice using same dose and duration of HCH treatment with an aim to know whether responses of antioxidant enzymes and extent of oxidative damages are similar or tissue specific.

MATERIALS AND METHODS

Chemicals

γ-HCH, thiobarbituric acid (TBA), catalase, glutathione reductase, cumene hydroperoxide (c-OOH), sephadex G-25, bovine serum albumin and 5,5'-dithiobis)-2nitrobenzoic acid (DTNB) were obtained from Sigma Chemical Company, USA. Tert-butyl hydroperoxide (t-Bu OOH) and EDTA were obtained from Merck-Schudant, Germany. Horse Radish peroxidase (HRP), NADPH, reduced and oxidized glutathione, L-methionine, riboflavin, 1-chloro-2,4, dinitrobenzene (CDNB) and xylenol orange were purchased from SISCO Research Laboratory, India. Hydroxylamine hydrochloride was obtained from Koch-Light Laboratory Ltd., England. All other chemicals used were of analytical grade unless and otherwise mentioned.

Animals and Pesticide Treatment

Adult male Swiss albino mice of age 90-120 days weighing about 25-35 g were maintained in poly-propylene cages (430 x 270 x 150 mm^3) in animal room of the Department as described earlier (Samanta and Chainy, 1997). Animals were randomly divided into 2 groups. Animals of Group II were administered γ-HCH (20 mg/kg/day, intraperitoneally) suspended in 0.1 ml of refined groundnut oil for 30 days. Animals of Group I served as the control and received the vehicle solution only. Mice from both groups were sacrificed 24 h after the last injection. Liver, kidney and cerebral cortex (CC) were quickly dissected out, washed free of blood with 0.9% (w/v) ice-cold saline and processed immediately for biochemical estimations.

Tissue processing

A 20% (w/v) homogenate was prepared in ice-cold phosphate buffer (50 mM, pH 7.4) containing 0.25 M sucrose in a motor driven homogenizer. The homogenate was centrifuged at 200 x g for 10 minutes at 4°C to precipitate cellular debris and the supernatant (crude homogenate) was used for the assay of lipid peroxides, hydrogen peroxide, water-soluble organic hydroperoxide and reduced glutathione (GSH). An aliquot of the supernatant was then subjected to centrifugation at 10,000 x g for 20 min at 4°C and the supernatant fraction was taken directly for the assay of catalase (CAT), whereas Sephadex G-25 elute of the 10,000 x g supernatant was used for assay of superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione S transferase (GST) activities.

Biochemical assays

Lipid peroxidation

Lipid peroxidation (LPX) in crude homogenate was estimated according to the method of Ohkawa et al., (1979) by monitoring the formation of thiobarbituric acid reactive substance (TBARS) formation. In order to find out the total inducible peroxidizability of the tissues, the samples were incubated with hydrogen peroxide (600 nmol/ mg protein); tert-butyl hydroperoxide (600 nmol/mg protein) and ferrous sulfate/ascorbic acid ($FeSO_4$/AA, 0.25mM/ 0.01mM) for one hour (Das and Chainy, 2001) at 37°C and formation of TBARS was monitored.

Hydrogen peroxide and water soluble organic hydroperoxide

Hydrogen peroxide (H_2O_2) and water soluble hydroperoxide (ROOH) contents of the crude homogenate of the tissues were estimated according to the methods of Pick and Keisari (1981) and Wolff (1994), respectively.

Reduced glutathione

Estimation of GSH content in the deproteinized crude homogenate was done according to the method of Sedlak and Lindsay (1968).

Enzyme assays

Activities of antioxidant enzymes such as superoxide dismutase (EC. 1.15.1.1), catalase (EC. 1.11.1.6), and glutathione peroxidase (EC. 1.11.1.9) were assayed as described earlier (Das and Chainy, 2001). Assay of glutathione S-transferase (EC.2.5.1.18) was done according to the method of Habig et al., (1974).

Protein content of samples were estimated according to the method of Lowry et al., (1951).

Statistics

Unpaired students' t test was performed to find the difference between control and experimental groups. A difference was considered statistically significant at $P < 0.05$ level.

RESULTS

Lipid peroxidation

The level of TBARS remained unchanged in the liver of HCH treated mice whereas its level decreased significantly in kidney and CC by 42% and 46% respectively. Although sensitivity to *in vitro* peroxidation assayed after incubation with various oxidants showed substantial increase in TBARS level in all tissues, the pattern varied from one tissue to the other (Table 1). When aliquots of liver homogenate were challenged extraneously with various oxidants maximum induction was noticed by incubation with $FeSO_4$ and ascorbic acid followed by *tert*-butyl hydroperoxide and the least was observed with H_2O_2. On the other hand, maximum induction in TBARS formation was recorded for *tert*-butyl hydroperoxide in the kidney and H_2O_2 failed to induce LPX in the tissue. In case of CC, maximum induction was noticed by $FeSO_4$ and ascorbic acid followed by H_2O_2 and *tert*-butyl hydroperoxide induced the minimum. Although the magnitude of induction of LPX by oxidant showed noticeable

difference, the pattern of response to HCH is more or less similar to that observed for endogenous LPX. However, $FeSO_4$ and ascorbic acid stimulated TBARS formation in the liver showed a significant elevation (78%) in response to HCH.

Hydrogen peroxide and water soluble organic hydroperoxide content

Hydrogen peroxide content remained unaltered in the hepatic tissue, declined significantly in case of kidney, but was elevated in CC in response to HCH treatment (Fig. 1). On the other hand, ROOH content of the liver augmented in response to HCH while that of CC was decreased. HCH failed to alter ROOH content of kidney (Fig. 2).

Antioxidant defences

A significant decrease in SOD activity in the liver (21%) and CC (37%) was noticed in response to HCH treatment whereas in case of kidney no change was recorded (Fig. 3). Although HCH exposure resulted in elevation of CAT activity in case of CC, it had no effect on the enzyme activity in liver and kidney (Fig. 4). Glutathione peroxidase activity was enhanced by 68% and 66% in case of liver and kidney, respectively, in response to HCH administration whereas the same treatment resulted in 34% decrease in the enzyme activity in case of CC (Fig. 5). An elevation of 45%, 27% (not significant) and 238% was noticed in GST activity of liver, kidney and CC in response to HCH administration, respectively (Fig. 6). A decrease of 26% and 44% in GSH content was recorded for liver and kidney, respectively in response to HCH treatment whereas no change was seen in CC (Fig. 7).

DISCUSSION

In general, the results suggest the differential distribution of antioxidant defence system in various tissues of mouse, which experience tissue specific differential modulation when exposed to HCH. The highest activities of antioxidant enzymes (SOD, CAT and GPX) are recorded in mouse liver followed by kidney and the least is observed in case of CC, which is in conformity with the findings for the same tissues of other mammals (Halliwell and Gutteridge, 2001).

Under normal physiological condition, antioxidant enzymes act in concert with each other to protect the tissues from noxious effects of reactive oxygen species and their metabolites. Superoxide dismutase dismutates superoxide radical ($O_2^{\cdot-}$) to H_2O_2, which is further neutralized to H_2O by either CAT or GPX. Therefore, it is obvious that any impairment caused to any member of the cascade will influence the activities of the other enzymes. In case of liver, a

decrease in SOD activity in response to HCH treatment will favour the accumulation of $O_2^{\cdot-}$ together with decreased generation of H_2O_2. In cellular systems H_2O_2 concentration is one of the deciding factors for CAT and GPX activities since both have a different Km value for the substrate; i.e. CAT: 25 mM for CAT(Nelson and Cox, 2001) and GPX: 1-10 mM for GPX(Barman, 1974). Lower intracellular concentration of H_2O_2 might be responsible for the failure of CAT induction in the liver. To corroborate the above findings the level of H_2O_2 was found to be at the basal level (same as in control animals) after HCH treatment. The augmentation in GPX activity in the liver could be an adaptive response to increased concentration of ROOH in pesticide treated animal liver since the enzyme is known to reduce lipidic and non-lipidic organic peroxides by utilization of two molecules of GSH (Flohe, 1982). Besides being a substrate for GPX, the tripeptide GSH also forms conjugates with electrophilic metabolites of HCH by the action of GST (Portig et al., 1979) which is further metabolized to mercapturic acid and released from cell (Habig et al., 1974). The increased hepatic GST activity and decreased GSH content in HCH treated mice are in total agreement with the above findings. Alternatively, GSH utilization for the effective removal of accumulated $O_2^{\cdot-}$ due to SOD depletion can not be ruled out (Wardman, 1988; von Sonntag and Schumann, 1990; Winterborn, 1995).

In the present study, the renal antioxidant enzymes of mouse were found to be highly resistant to HCH treatment except for GPX whose activity was augmented by 66% in response to the pesticide treatment. The decrease in H_2O_2 and GSH level in the pesticide treated animals could be attributed to the increased utilization of both the substrates by the elevated GPX activity in HCH treated mice.

The scenario of oxidative stress parameters in the CC of mice in response to HCH is totally different from the other two tissues where a significant decline in SOD and GPX activity was observed along with a sharp elevation in cerebral CAT activity. To neutralize the enhanced H_2O_2 level in the CC of mice in response to HCH exposure and to compensate decreased GPX activity, CAT activity was probably enhanced in the CC. Although the decline in SOD level will favour $O_2^{\cdot-}$ accumulation and retard H_2O_2 generation from its dismutation a sharp increase in cerebral H_2O_2 level was noticed in response to HCH. Since in the present study ascorbic acid content in the cerebral tissue was not measured, the contribution of its oxidation by $O_2^{\cdot-}$ to H_2O_2 can not be ignored. It is reported that $O_2^{\cdot-}$ oxidizes ascorbic acid into ascorbate

radical with the formation of H_2O_2 at physiological pH (pH 7.4, $K_2 = 2.7 \times 10^5 M^{-1} s^{-1}$, Halliwell and Gutteridge, 2001).

The findings of the present study, demonstrates a decline in the level of TBARS (an index of tissue lipid peroxidation) in kidney and CC without any significant alteration in its level in the hepatic tissue. The results contradict the previous findings with rat liver (Arisi *et al.*, 1994) and brain (Hincal et al., 1995; Sahoo et al., 2000) where significant elevations in TBARS levels were observed in response to the pesticide treatment. The response of various biochemical components of tissues to HCH treatment differs from species to species. The levels of phospholipids and triglycerides are reported to be elevated in the liver of rats in response to HCH (Srinivasan and Radhakrishnamurty, 1989) whereas in case of mice the level of triglycerides was depleted without affecting the phospholipid composition (Ravinder et al., 1990). Since phospholipid and fatty acid composition are two important factors affecting tissue lipid peroxidation (Halliwell and Gutteridge, 2001), the decrease in TBARS level in mice tissues could be due to alteration in their lipid profile in response to the pesticide.

The observed tissue specific response of oxidative stress parameters is not unexpected in the context of variable response of antioxidant defences recorded for HCH in different tissues of other species. Barros et al., (1991) demonstrated a significant increase in the activities of SOD and CAT along with microsomal $O_2^{\cdot-}$ level without any change in levels of glucose-6-phosphate dehydrogenase, GPX and glutathione reductase in response to a- or γ-HCH in gavage. On the other hand, the above group failed to observe any change in oxidative stress status of the brain of rats in response to acute and short-term administration of γ-HCH (Arisi et al., 1994). Again in another study, the authors observed augmentation in levels of GSH, GPX and glutathione reductase (GR) accompanied by a fall in CAT activity in the liver in response to lindane (γ-HCH, 20 mg/kg) treatment for 3 consecutive days. No change in SOD activity of liver was recorded (Junqueira et al., 1994). The authors also noticed increase in SOD activity and GSH level in erythrocytes without any change in its CAT and GPX activities. In an earlier study from our laboratory, we have reported induction of oxidative stress in the liver of chick by HCH, where an increase in the levels of GSH and TBARS in various hepatic subcellular fractions was noticed without any significant alteration in the activities of SOD and CAT (Samanta and Chainy, 1995). In another study in rats, it was demonstrated that, the same treatment schedule (20 mg/kg) elicited an increase in LPX, GR and ascorbic acid level in the brain concomitant with a decline in the levels of SOD, CAT,

GPX and GSH (Sahoo et al., 2000). In a recent report on testicular oxidative stress parameters of mouse and rat, we observed species-specific variation in response to HCH treatment (Samanta and Chainy, 2002). Thus, it is apparent that response of antioxidant defences to HCH is dependent on the nature of the tissue as well as the species concerned.

FIGURE LEGENDS

Figure 1.Effect of HCH (20 mg/kg body wt./day for 30 days, ip.) on H_2O_2 content (nmol/mg protein) of mouse tissues. Data are expressed as mean ± s.d. of 5 observations. *p<0.05 in comparison to respective control.

-Control, -HCH treated.**

Figure 2.Effect of HCH (20 mg/kg body wt./day for 30 days, ip.) on water-soluble organic hydroperoxide content (nmol/mg protein) of mouse tissues. Data are expressed as mean ± s.d. of 5 observations. *p<0.05 in comparison to respective control.

Figure 3.Effect of HCH (20 mg/kg body wt./day for 30 days, ip.) on cytosolic superoxide dismutase (SOD) activity (Units/mg protein) in mouse tissues. Data are expressed as mean ± s.d. of 5 observations. *p<0.05 in comparison to respective control.

Figure 4.Effect of HCH (20 mg/kg body wt./day for 30 days, ip.) on catalase activity (pkat/ mg protein) in mouse tissues. Data are expressed as mean ± s.d. of 5 observations. *p<0.05 in comparison to respective control.

Figure 5..Effect of HCH (20 mg/kg body wt./day for 30 days, ip.) on cytosolic glutathione peroxidase activity (nmol NADPH oxidized/min/mg protein) in mouse tissues. Data are expressed as mean ± s.d. of 5 observations. *p<0.05 in comparison to respective control.

Figure 6.Effect of HCH (20 mg/kg body wt./day for 30 days, ip.) on cytosolic glutathione S-transferase activity (nmol CDNB conjugated/min/mg protein) in mouse tissues. Data are expressed as mean ± s.d. of 5 observations. *p<0.05 in comparison to respective control.

Figure 7. Effect of HCH (20 mg/kg body wt./day for 30 days) on glutathione (GSH) content (nmol/g tissue) of mouse tissues. Data are expressed as mean ± s.d. of 5 observations. *p<0.05 in comparison to respective control.

Fig. 1

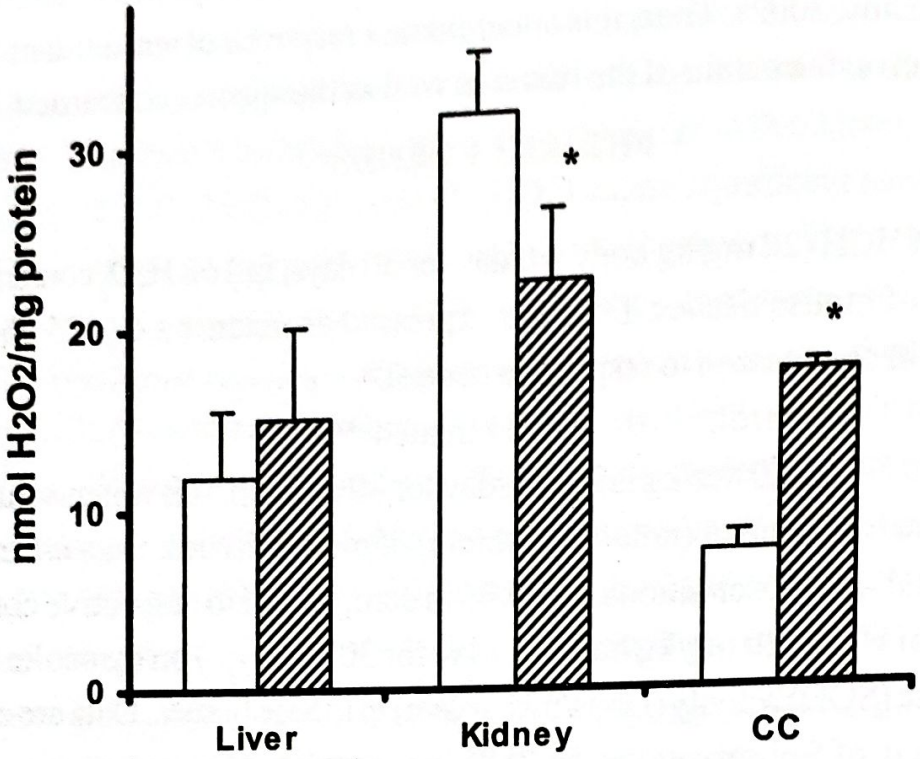
nmol H2O2/mg protein
30
20
10
0
*
*
Liver
Kidney
CC

Fig. 2

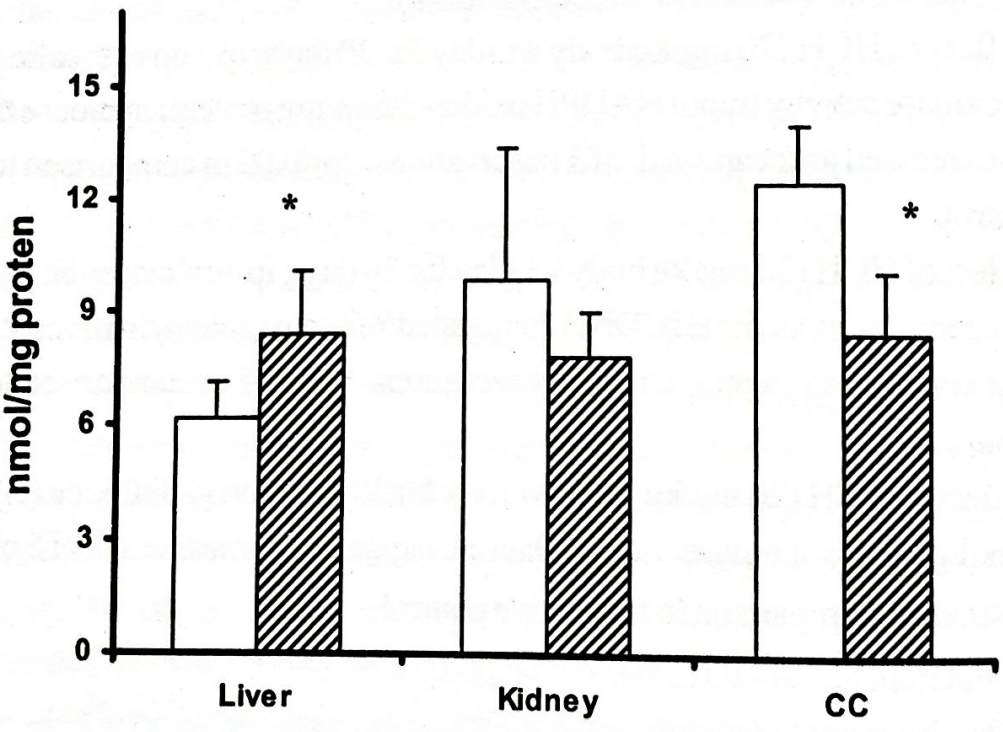
nmol/mg proten
15
12
9
6
3
0
*
*
Liver
Kidney
CC

Fig. 3

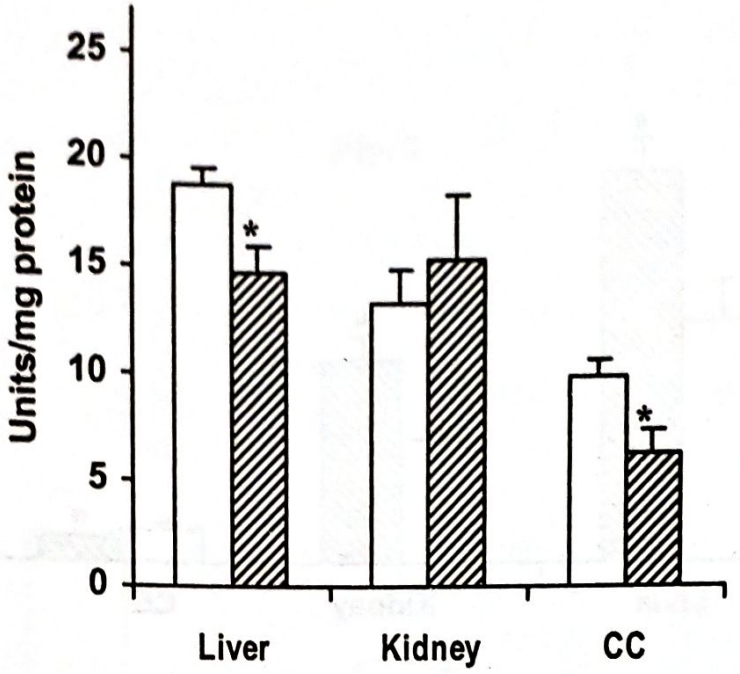
Units/mg protein
25
20
15
10
5
0
*
*
Liver
Kidney
CC

Fig. 4

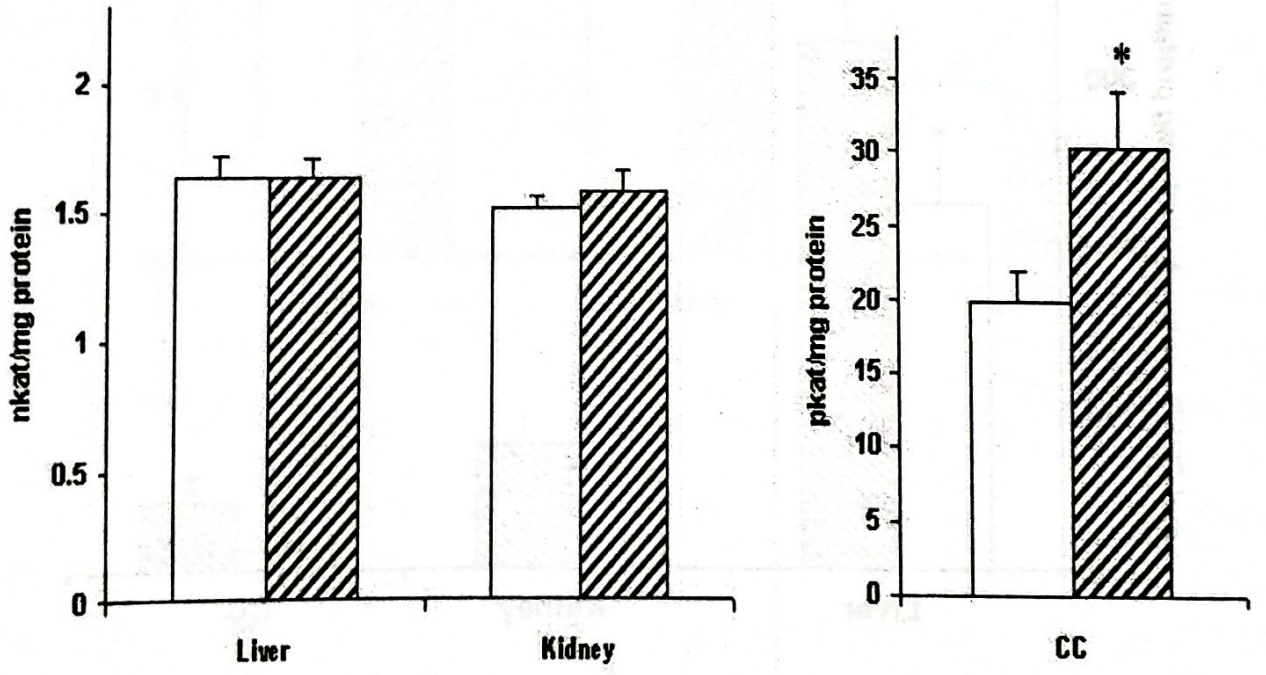
nkat/mg protein
2
1.5
1
0.5
0
Liver
Kidney
pkat/mg protein
35
30
25
20
15
10
5
0
*
CC

Fig. 5

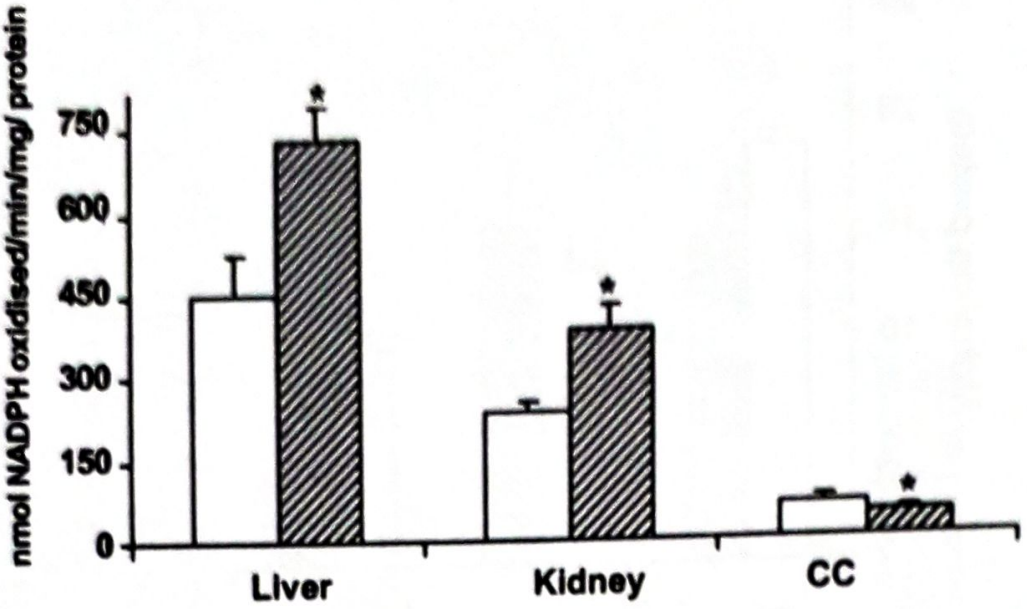
nmol NADPH oxidised/min/mg/ protein
750
600
450
300
150
0
Liver
Kidney
CC

Fig. 6

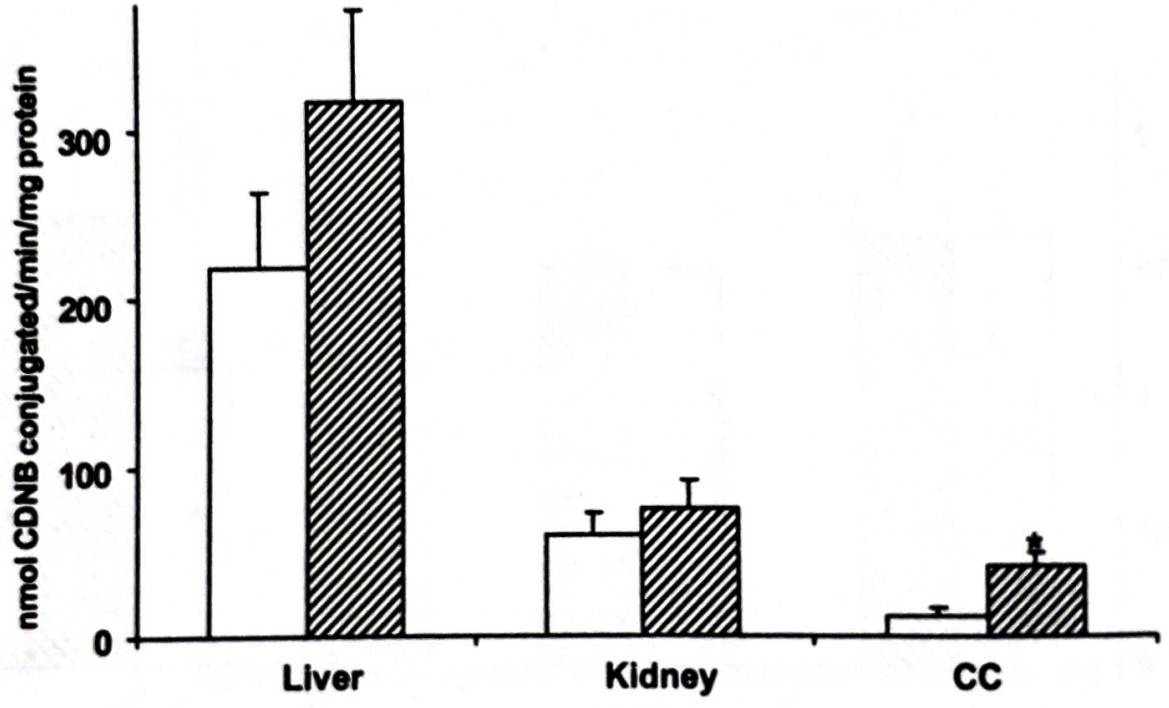
nmol CDNB conjugated/min/mg protein
300
200
100
0
Liver
Kidney
CC

Fig. 7

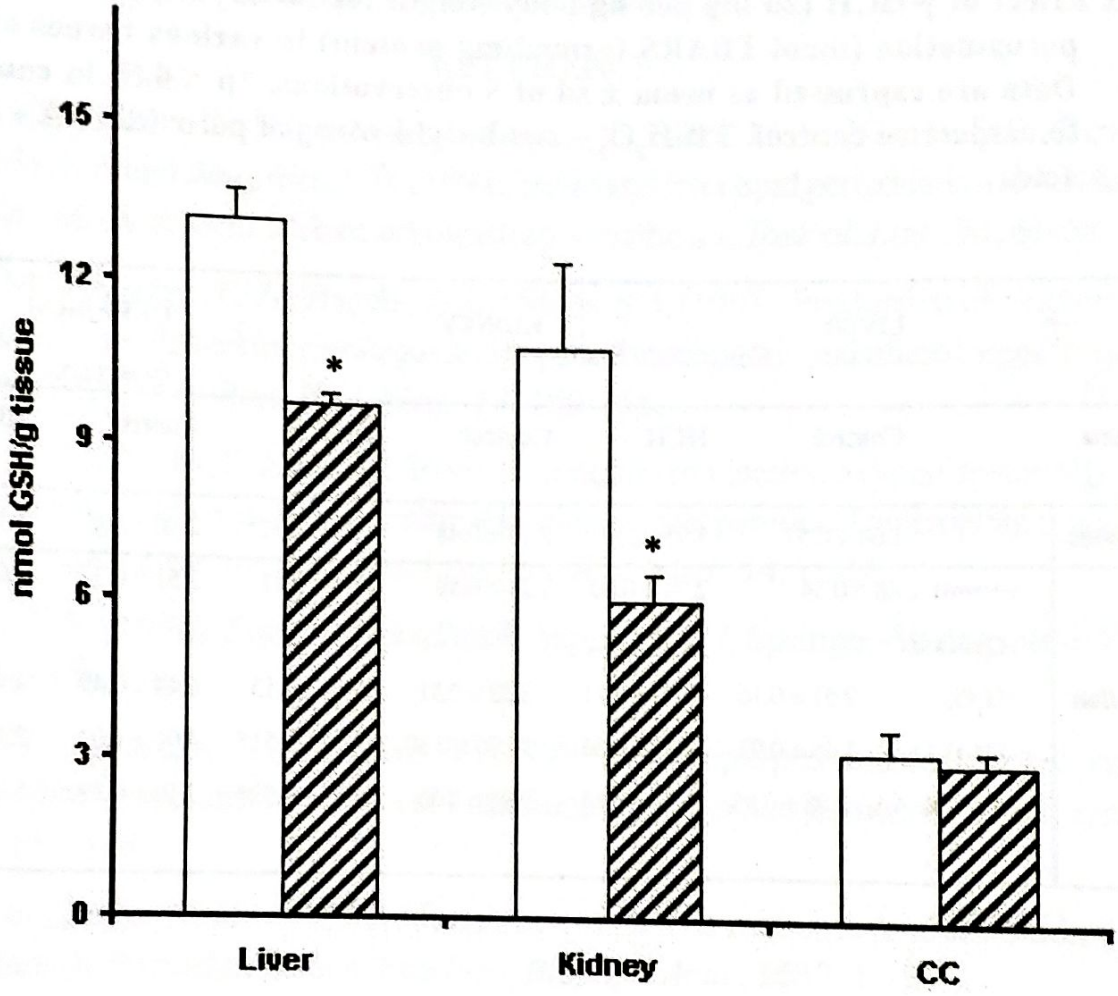

Fig. 7

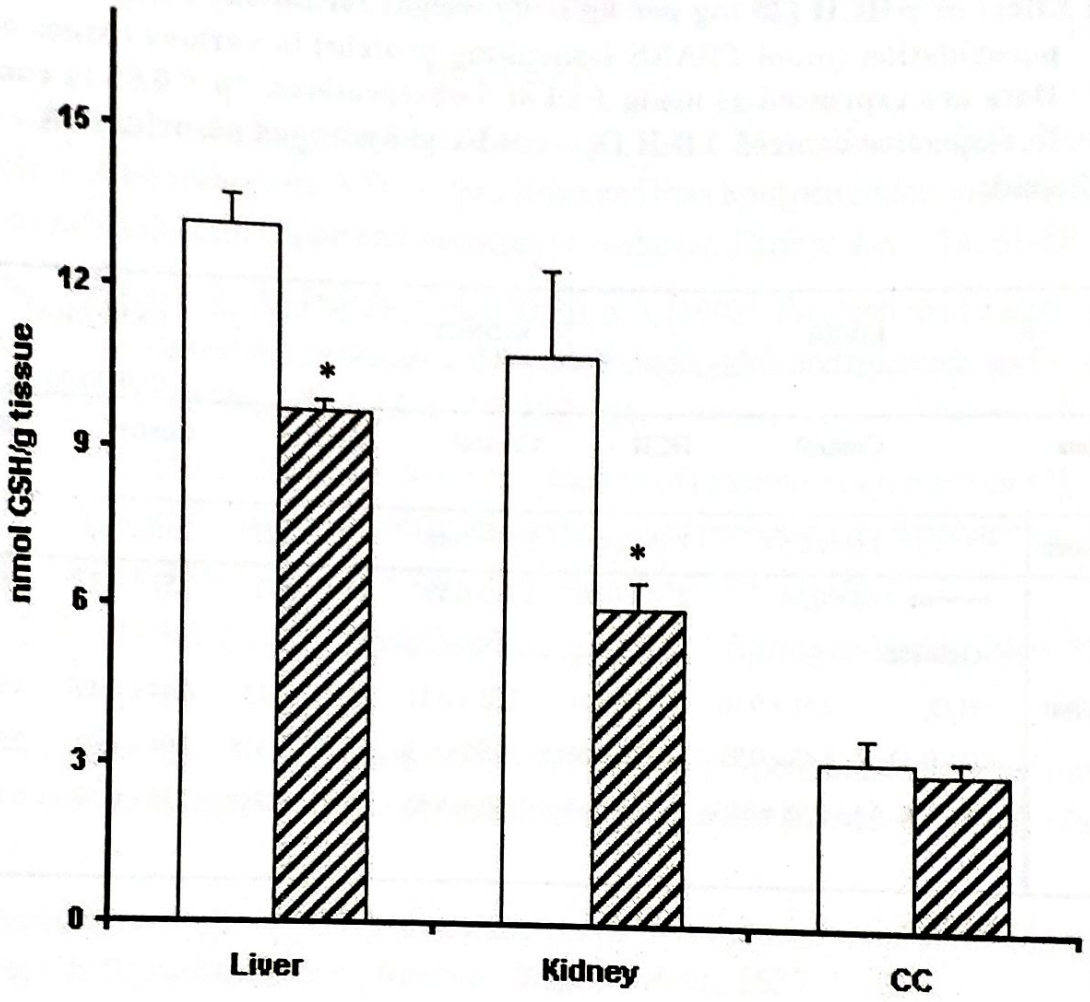

Table-1 Effect of γ-HCH (20 mg per kg body weight for 30 days, ip) on level of lipid peroxidation (nmol TBARS formed/mg protein) in various tissues of mouse. Data are expressed as mean ± sd of 5 observations. *p < 0.05 in comparison to respective control. TB-H_2O_2 – *tert*-butyl hydrogen peroxide, AA – ascorbic acid.

Tissues →	LIVER		KIDNEY		CEREBRAL CORTEX	
Conditions ↓	Control	HCH	Control	HCH	Control	HCH
Endogenous	1.68 ± 0.57	1.75±0.40	2.16 ± 0.48	1.25 ± 0.27*	2.10 ±0.68	1.14 ± 0.38*
+Incubation -Oxidant	2.48 ± 0.34	2.74 ± 0.60	2.23 ± 0.59	1.44 ± 0.11	2.51 ± 0.33	1.61 ± 0.69
+H_2O_2	2.51 ± 0.16	2.60 ± 0.11	2.33 ± 0.51	1.71 ± 0.13	6.44 ± 0.49	4.46 ± 0.82*
+TB-H_2O_2	5.45 ± 0.93	4.87 ± 0.66	10.96 ± 0.30	7.11 ± 0.51*	4.01 ± 1.03	2.18 ± 0.77*
+$FeSO_4$ & AA	2.98 ± 0.91	5.31±0.91*	9.81 ± 1.63	5.37 ± 0.98*	7.16 ± 0.19	5.17 ± 0.51*

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TAIL REGRESSION IN THE TADPOLES OF *BUFO MELANOSTICTUS*

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ABSTRACT

In the present study, morphological and histological aspects of the regressing tail of *Bufo melanostictus* has been studied. There was gradual reduction in tail length from stage III to stage XXIV, where the flat and elongated tail is reduced to a stump. Histological analysis has revealed shortening of tail fins and thickening of tail epidermis as the characteristic feature of regressing tail. Disintegration of muscle bundles and notochord is more at the distal end than proximal end, which shows that regression begins from the distal end and proceeds proximally. With the degeneration of muscles, notochord, nerves and blood vessels, appearance of a large number of melanocytes around the disintegrating tissues suggests that the melanocytes play some role in disintegration apart from imparting pigmentation to the skin.

Key words: Tail regression, *Bufo melanostictus.*

INTRODUCTION

Tail regression is the most spectacular event that occurs during anuran metamorphosis. Metamorphosis is also a dramatic example of extensive morphological, biochemical and cellular changes occurring during post-embryonic development (Tata, 2003). The aquatic anuran tadpoles metamorphose to the terrestrial adult frog through a complex programme that results from changes in gene expression. The metamorphic changes of frog development are brought about by hormones thyroxine (T_4) and triiodo thyronine (T_3) from the thyroid during metamorphosis (Kistler et al., 1977 and Robinson et al., 1977). In amphibian

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metamorphosis, different tissues or group of cells within the same tissue can exhibit different hormonal responses, which range as widely as *de novo* metamorphosis, functional reprogramming and total tissue regression (Tata, 2003).

The degeneration of tail structure during anuran metamorphosis is relatively rapid, as the bony skeleton does not extend into the tail (Wassersug, 1989). The regression of tail is an excellent example of apoptosis or programmed cell death. The tail, which accounts for one third of the body weight, is completely resorbed in a few days during metamorphic climax (Kerr et al., 1974). Tail tissues are concurrently resorbed throughout the tail, resulting in the graded rate of shortening of tissue length, increasing distally (Dymtrenko et al., 1981). Apoptosis involves the death of cell followed by phagocytosis of cell debris by macrophages and other cells (Kerr et al., 1974 and Gilbert and Freiden, 1981). In all anuran species so far studied, the retraction of tail fins has been found to represent the first sign of tail atrophy and reduction of tail length becomes discernible later. In the connective tissue of tail fins, there is gradual disappearance of ground substance as well as the fibrillar components viz. collagenous and argyrophil fibres, respectively. The connective tissue between the myomeres disintegrate, the muscle fibre show fragmentation and the nerve fibre also degenerate. Nerve degeneration begins from the periphery (Weber, 1968).

Since anuran tail is one of the best models to understand programmed cell death, in the present study an attempt has been taken to understand the mechanism underlying tail regression in the common Indian toad *Bufo melanostictus*. The histological and morphological aspects of pre-regressing and regressing tail have been investigated in the present study.

MATERIALS AND METHODS

Tadpoles

Egg masses of common Indian toad *Bufo melanostictus* were collected from Kakhadi, Athagarh of Cuttack district in Orissa (20° 30' 3" N 85° 51' 55" E) during the month of March of the year 2006. For investigation, five stages of tadpoles i.e. tadpoles in hind limb bud stage (Stage III), well-developed hind limbs (Stage XVIII), both forelimbs emerged (Stage XXI), more than half of the tail regressed (Stage XXIII) and tail as stump (Stage XXIV) were selected (Stages according to Taylor and Kollros, 1946).

Rearing of tadpoles and tail amputation

Tadpoles were reared in the laboratory following the standardized procedure (Mohanty – Hejmadi, 1977). Tap water was collected and stored for 72 hours for conditioning. The conditioned tap water was used for the rearing of the tadpoles. The tadpoles were fed with boiled *Amaranthus* leaves and boiled egg yolk *ad libitum* throughout rearing. For investigation, five different stages of tadpoles i.e. Stage III, Stage XVIII, Stage XXI, Stage XXIII, Stage XXIV (Stages according to Taylor and Kollros, 1946) were selected. The tadpoles were anesthetized with MS 222 (Tricaine methane sulphonate) prior to the tail amputation. Tail amputation was done by keeping the specimens laterally on a pre sterilized porcelain plate. With the help of a sharp sterilized blade, tail was amputated through the base.

Following amputation, stage III and XVIII tadpoles were kept in conditioned water whereas stage XXI, XXIII and XXIV tadpoles were kept in an amphibious condition for further development. On completion of metamorphosis, the toadlets were released in nature at the vicinity of collection of egg.

Morphological investigation of regressing tail

For morphological investigation five different stages i.e. III, XVIII, XXI, XXIII and XXIV tadpoles were selected. A pool of five tadpoles was taken and the snout to tail tip (STT) length of the tadpoles was measured in mm.

Histological investigation of regressing tail

For histological investigation, three stages i.e. stage III, stage XXIII and stage XXIV tadpoles were taken. Following tail amputation, the tail tips were fixed in Bouin's fixative for 48 hours. The tail pieces were kept in running water overnight for complete removal of Bouin's fixative. Then the tails were dehydrated through a graded series of alcohols i.e. 30%, 50%, for 10 minutes each and then kept in 70% alcohol overnight. After overnight dehydration in 70% alcohol, the tails were kept in 90% and absolute alcohol for 30 minutes each. After complete dehydration, the dehydrated tails were transferred to a mixture of 50% xylene and 50% paraffin wax (melting point 58°C) and kept in an oven for 30 minutes. For proper embedding tails were subjected to two more changes of paraffin wax for 30 minutes each. Blocks were prepared using metal L- blocks and paraffin embedded tails were transferred to the metal blocks containing molten paraffin wax. All the prepared blocks were left overnight. The

following day blocks were trimmed and mounted on the block holder. Transverse sections of the tail pieces of 5μ thickness were cut using a rotary microtome machine.

Clean glass slides were taken and a drop of egg albumin was uniformly applied as a coating on one surface of the slide. The slides were left for drying for 15-20 minutes. Then the tissue sections were placed on the albumin coated side of the slides. Drops of water were put on the slides and the slides were placed on hot plate for 2-3 minutes to stretch the wax sections on the slides. The stretched sections were left overnight to fix the tissues on the slides. The sections were then stained following Mallory's triple staining method.

The sections were deparaffinized with two changes of xylene and hydrated through a downgrade series of alcohols (i.e. 100%, 90%, 70%, 50% and 30%). The sections were stained in Mallory I for 10 minutes and then rinsed in distilled water to differentiate red. Then the sections were treated with phosphomolybdic acid for 10 minutes followed by staining with Mallory II for 15 minutes. Then the slides were rinsed in distilled water. The sections were treated with 90% ethyl alcohol to differentiate aniline blue. The sections were dehydrated in absolute alcohol by dipping them 3-4 times followed by mounting with DPX.

Photographs of clearly stained sections were taken using a compound microscope (Hund, H500) and Pentax camera.

RESULTS

Morphological analysis

The stage III tadpoles (Fig. 1A) with hind limb bud measured 11.7 ± 0.25 mm in snout to tail tip (STT) length. There was increase in size of the stage XVIII tadpoles with well-developed hind limbs, which measured 18.4 ± 0.577 mm in STT (Fig.1B). The STT was 18.3 ± 0.894 mm in the stage XXI tadpoles in which both forelimbs were emerged (Fig.1C). The STT of stage XXIII tadpoles was 10.6 ± 0.975 mm where more than half of the tail was regressed (Fig.1D). With further regression of tail, the STT of stage XXIV tadpoles was 8.8 ± 0.833 mm with tail as a stump (Fig.1E). A cumulative account of STT is given in table 1.

Histological analysis

Transverse sections (TS) of tail of the proximal end of stage III tadpoles (Fig.1F) showed distinct notochord (N) with thin notochordal sheath (NS). Nerve cord (NC) was present on

the mid-dorsal part above the notochord. Two distinct blood vessels (BV) were present mid-ventral to the notochord. Muscle bundles (MB) were arranged laterally and there was a thin epidermal layer (E) covering the muscle bundles. The dorsal (DTF) and the ventral tail fins (VTF) were present on the dorsal and ventral side, respectively. The tail fins were thin. Compact muscle bundles (MB) were present laterally below epidermal layer. A thin basement membrane was observed below the epidermal layer.

Towards the distal end, tail histology showed similar structures as observed for the proximal part. However, the tail sections were thinner with patches of muscle bundles bilaterally. The epidermal layer was thinner with a thin basement membrane. Both dorsal and ventral tail fins were thin and elongated. The distal sections became gradually thinner towards the tip. However, the arrangement of notochord, nerve cord, muscle bundles and tail fins remained the same except the distal most part where muscle bundles were indistinct.

In the post-metamorphic tadpoles of stage XXIII, the proximal most part of the tail showed a remarkable decrease in the length of both dorsal and ventral tail fins (Fig.1 G). Degenerated muscle bundles (DM) were seen in between compact muscle bundles (MB). The nerve cord (NC) and notochord (N) were distinct. The notochordal sheath (NS) was thicker. Mesenchymal cells (MC) were present at the base of both dorsal and ventral tail fins as well as in the fins. The epidermal layer (E) covering the tail fins was thicker with a cuticular layer (C) and a prominent basement membrane (BM). Sections taken from the distal part of tail showed similar structures but there was gradual decrease in the width of the tail, number of muscle bundles became less and the epidermal layer became thick and wavy with a prominent basement membrane. At the distal part of the tail all muscle bundles were disintegrated and notochordal sheath was thicker. Melanocytes were seen among the degenerating muscles. Blood vessels had started disintegrating and the blood cells were dispersed around the tail fins. The tail became thinner towards the more distal part. The notochordal sheath disintegrated and melanocytes appeared around the notochord and among the degenerating muscle bundles. Loose blood cells were seen in between the mesenchymal cells. Nerve cord was indistinct. At the distal most part there was complete disintegration of blood vessels showing loose blood cells. Melanocytes appeared on the periphery of disintegrating notochord. The width of the tail decreased with a thick epidermis and basement membrane. Mesenchymal cells were found within and at the base of the tail fins.

In the longitudinal sections of the tail of stage XXIII a clear representation of degenerating muscle bundles could be seen (Fig. 2A,B, C). Intact muscle bundles (MB) were found towards the proximal end of the tail whereas degenerating muscle bundles (DM) were seen towards the distal end. At the tail tip (TT) there were abundant mesenchymal cells (MC) along with scattered blood cells (BC). The epidermis (E) was thicker and wavy and lined by a thin basement membrane (BM)(Fig. 2C). The notochord (N) was lined by a wavy notochordal sheath (NS). Melanocytes (M) were found close to the disintegrating notochord (N)(Fig.2A). In the middle portion of the tail, notochord (N) was lined by a wavy notochordal sheath (NS)(Fig.2A). The epidermis (E) was thinner at this part of the tail than the tail tip and lined by a thick basement membrane (BM). Melanocytes (M) were seen around the degenerating muscle (DM) (Fig. 2B) Patches of muscle bundles (MB) along with degenerating muscles (DM) were also seen (Fig. 2A). Towards the proximal end, notochord was lined by a straighter notochordal sheath (NS). Compact and degenerating muscles (DM) were seen in this region. The epidermis (E) was thinner with a thick basement membrane (BM). Notochordal sheath (NS) was straight. No melanocytes (M) were seen (Fig. 2A).

In longitudinal sections of the tail of stage XXIV tadpoles, no muscle bundles were seen. Patches of degenerated muscles were seen at the proximal end. Abundant melanocytes were found at the tail tip as well as around the disintegrating notochord. The epidermis was thicker at the tail tip with thick basement membrane. However, at the proximal end, the epidermis was thinner than the tail tip with a thin basement membrane.

Table1. Snout to tail tip (STT) length of different developmental stages of *Bufo melanostictus*.

STAGES	STT (in mm)
Stage III † (Hind limb bud)	11.7 ± 0.25*
Stage XVIII (Well developed hind limb)	18.4 ± 0.577
Stage XXI (Forelimbs emerged)	18.3 ± 0.894
Stage XXIII (More than half of the tail regressed)	10.6 ± 0.975
Stage XXIV (Tail as stump)	8.8 ± 0.833

† = Stages according to Taylor and Kollros, 1946

* = Standard deviation

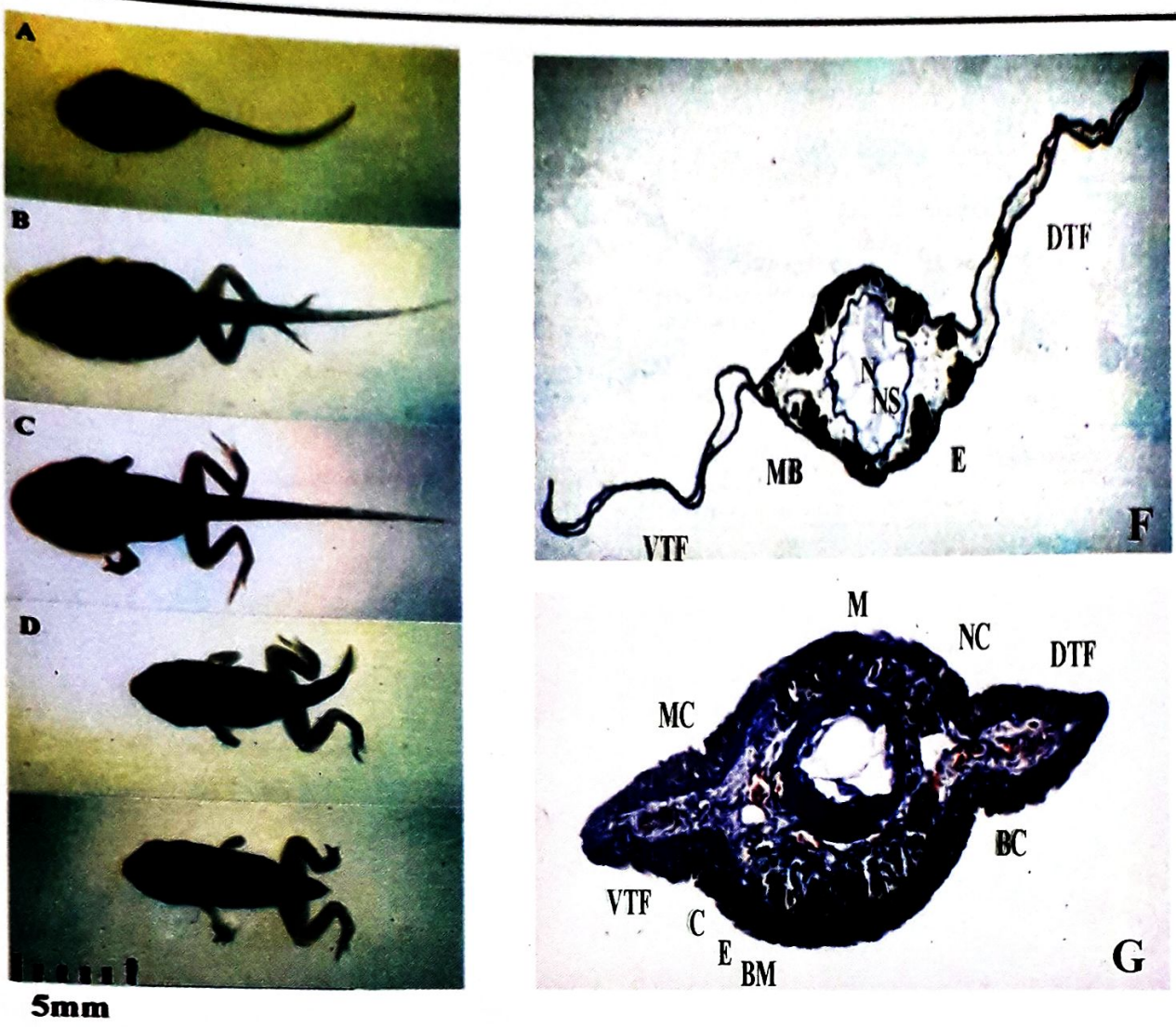

Fig 1. Various developmental stages of *B. melanostictus* and cross-section through the tails.

(A) Hindlimb bud stage (Stage III)
(B) Hindlimb well-developed (Stage XVIII)
(C) Forelimbs emerged (Stage XXI)
(D) Half of the tail regressed (Stage XXIII)
(E) Toadlets with tail as a stump (Stage XXIV)
(F) T.S. through base of tail of stage III showing distinct notochord (N), thin noto chordal sheath (NS), nerve cord (NC), distinct muscle bundles (MB), blood vessels (BV), thin epidermis (E) and elongated dorsal (DTF) and ventral tail fins (VTF) (100x)
(G) T.S. through base of tail of stage XXIII tadpoles showing degenerated notochord (N), thick notochordal sheath (NS), nerve cord (NC), degenerated muscle bundles (DM), mesenchymal cells (MC), loose blood cells (BC), melanocytes (M), thick epidermis (E)lined by a distinct basement membrane (BM)internally and cuticular layer (C) externally. the dorsal (DTF) and ventral (VTF) tail fins were thick and short with thick epidermal layer. (100X)

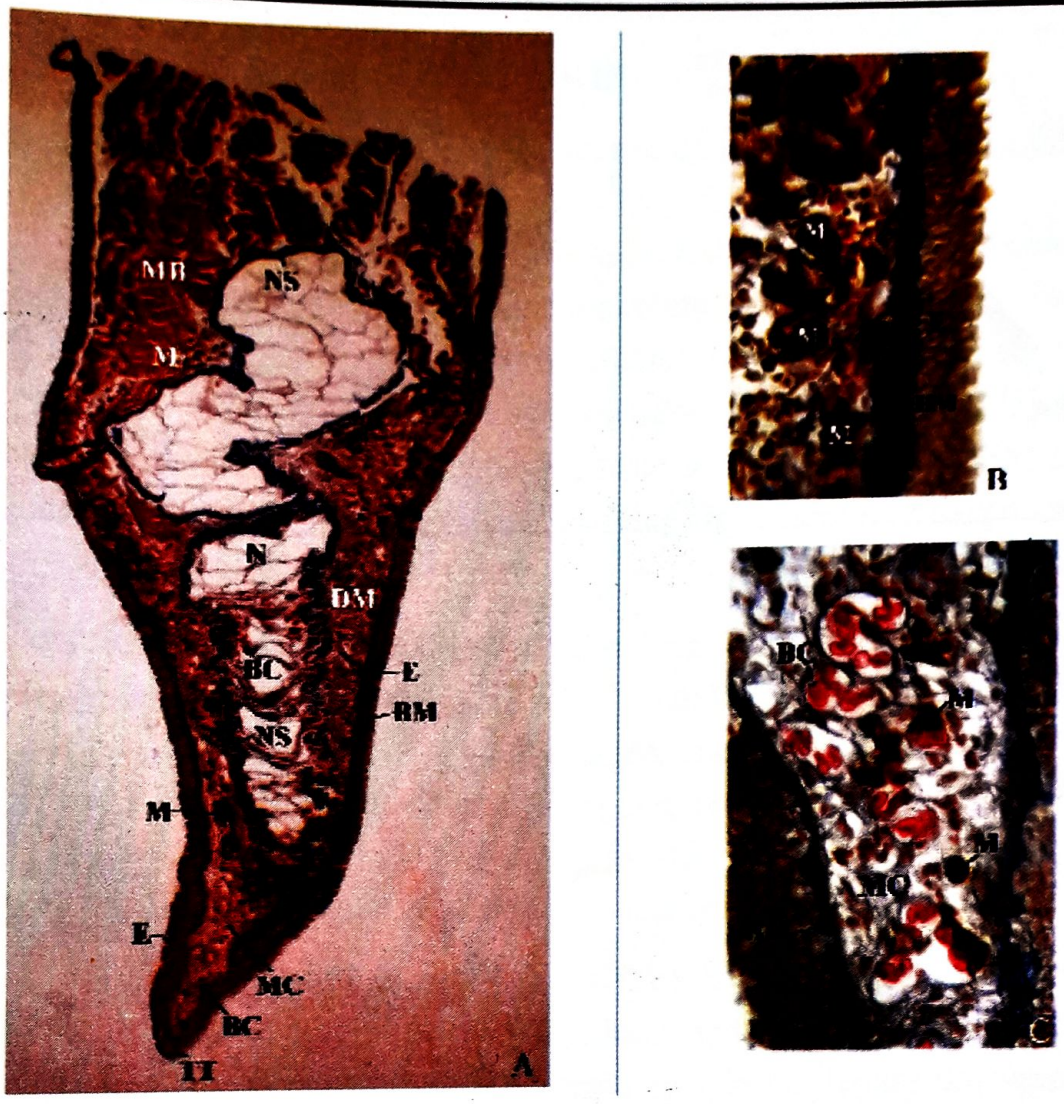

Fig. 2 L.S. through the tail of stage XXIII tadpole of *B. melanostictus.*

(A) A complete tail showing compact muscle bundles (MB) at the proximal part, degenerated muscle bundles (DM) at the distal part, disintegrating notochord (N), wavy notochordal sheath(NS) at the distal end, loose blood cells (BC), thick epidermis (E) with distinct basement membrane (BM) and melanocytes (M) at the distal end of the tail. (160x)

(B) Magnified portion of the tail through the proximal part showing multi-layered epidermis (E), thick basement membrane (BM) and melanocytes (M). (800x)

(C) Magnified portion of tail through distal part showing loose blood cells (BC), mesenchymal cells (MC), melanocytes (M), thin basement membrane (BM) and a thick epidermal layer(E).(800x)

DISCUSSION

Maximum size (STT) was observed in the tadpoles of stage XVIII. They were 1.628 times longer than the tadpoles of stage III. There was gradual reduction in size in the tadpoles of stages XXI, XXIII and XXIV. The STT length was reduced by 1.005 times in the stage XXI tadpole. The stage XXIII tadpole showed 1.735 times reduction in size than the stage XVIII tadpoles. However, stage XXIV toadlets were 2.09 times shorter than the stage XVIII tadpoles. The reduction in size was due to reduction in tail length. Reduction in tail length has been reported for several species of anurans (Sasaki et al., 1988) following onset of metamorphosis at stage XX. So reduction in STT of stage XXI tadpole in the present study favors the report of Sasaki et al., 1988.

Histological analysis of pre-metamorphic tadpole tails (Stage III) showed a distinct notochord with thin notochordal sheath. Well-organized muscle bundles were present laterally. Both dorsal tail fin and ventral tail fin were thin and elongated. The outer epidermal layer was thin with a narrow basement membrane. Arrangement of tail tissue remained the same from proximal to distal end of the tail. However, the distal most part of the tail was thinner than the proximal and middle part of the tail with scanty or no muscle bundles.

The post-metamorphic tadpole (Stages XXIII and XXIV) showed marked differences in the tail histology. There was reduction in length of both dorsal and ventral tail fin. The epidermal layer became thick. It was lined by a distinct basement membrane. It has been reported that the basement membrane between the epidermis and dermis of the tail thickens during metamorphosis (Usuku et al., 1965). Elimination of tail skin during metamorphosis has also been described to be dermis dependant (Kinoshita et al., 1986). Towards the distal end, the epidermal lining became wavy. A distinct cuticular layer was found outside the epidermal layer. Wavy and thick epidermal layer has been described as the characteristics feature of anuran tail epidermis during regression (Watanbe et al., 2001). It has been reported that the tail consists of several major tissue types. These tissues are separated from each other by various extra cellular matrices (ECM's). The expression of matrix metalloproteinases (MMP's) suggests that three different MMP's i.e. stromelysin (ST3), collagenase 3 (Col 3) and collagenase 4 (Col 4) are involved in the resorption of different tissues during development (Damajanovski et al., 1999). In the present study notochord was covered by a distinct notochordal sheath throughout the tail in the pre-metamorphic tadpoles. In the post-

metamorphic tadpoles thickening of the notochordal sheath was observed which was followed by disintegration of the notochordal sheath from distal end of the tail. The notochordal sheath is made of collagen and has been reported to be eliminated during tail resorption. Two matrix metalloproteinases i.e. collagenase 3 (Col 3) and collagenase 4 (Col 4) has been described to be expressed in the notochordal sheath. Col 3 is expressed in the entire notochordal sheath while Col 4 is expressed in the inner cells of notochordal sheath (Berry et al., 1998 and Damajanovski et al., 1999). Present investigation has shown muscle bundle disintegration to be the maximum at the distal end. Patches of muscle bundles were found in the proximal part of the tail along with degenerated muscle fibres. It has also been reported that the most abundant tissue in the tail is muscle and it is also eliminated through apoptosis (Watanbe and Sasaki, 1974; Kerr et al., 1974 and Nishikawa and Hayashi, 1995) and appears to involve cell death regulators like caspases and Bax/Bcl-2 family members (Sachs et al., 1997 and Yaiota and Nakajima, 1997). The matrix metalloproteinase stromelysin-3 (ST3) was found to be expressed in the fibroblasts surrounding the muscle flank whereas Col 3 and Col 4 were absent in the muscle as well as fibroblasts (Berry et al., 1998 and Damajanovski et al., 1999). The longitudinal sections of stage XXIII tadpoles showed the accumulation of loose blood cells at the tail tip. There was thick epidermis with thin basement membrane at the tail tip but a thin epidermis with a thick basement membrane towards the proximal end. Melanocytes appeared at the distal end in between the tail epidermal layers and at the base of the tail fin. Melanocytes were abundant and mainly found in and around the degenerating muscle fibres and disintegrating notochord. In stage XXIV tadpoles, large numbers of melanocytes were found to accumulate at the tail tip as well as around the degenerating notochord. The number of melanocytes in the dermis has been found to increase in the regressing tail and it confirms the report that the increase in number of melanocytes is due to the migration of the epidermal melanocytes to the dermis (Yasutomi, 1987).

The presence of degenerating notochordal cells with a wavy notochordal sheath was observed in the distal end whereas an intact notochord was found at the proximal end. Further the muscle degeneration was more at the distal end than the proximal end. Presence of loose blood cells at the tail tip and in the tail fins indicated the disintegration of blood vessels. The blood cells were not only scattered around the tail tip and regressing tail fins but also entered into the degenerating notochord. From the histological investigation it can be concluded that tail regression began at the distal end of the tail and extended towards the proximal end.

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GENOTOXIC EFFECT OF GENTAMICIN AND TOBRAMYCIN ON MEIOTIC CHROMOSOMES OF *POECILOCERUS PICTUS* F. (INSECTA: ORTHOPTERA :ACRIDIIDAE)

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ABSTRACT

The long term exposure of humans to different man - made drugs leads to several health hazards of which genotoxicity is of prime importance. The investigation on the effect of two aminoglycoside compounds namely Gentamicin and Tobramycin on grasshoppers shows that both of these compounds have induced chromosomal aberrations significantly with increase in concentration and duration of exposure to these compounds. Hence, careful and controlled use of the stated drugs is suggested.

Keywords: Gentamicin, Tobramycin, *Poecilocerus pictus* F, Chromosomal aberration.

INTRODUCTION

For the curing of various diseases, drugs are used to a great extent. However, these drugs most often act as mutagens in our tissues (Auerbach and Robson, 1944; Kost, 1949; Somers and Hu, 1962; Palmer et al., 1972; Pati and Bhunya, 1987; Spencer et al., 1994) and cause mutation in humans. The drugs like Gentamicin and Tobramycin are two aminoglycoside compounds, which are used as antibiotics. To assess the effect of such drugs, an investigation was undertaken *in vivo* to study the direct effect of Gentamicin and Tobramycin on short horned grasshopper *Poecilocerus pictus*.

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MATERIALS AND METHODS

For this investigation, the experimental animal was short-horned grasshopper (*Pocilocerus pictus* F.), which were collected from different regions of Bhubaneswar (20.15° N and 85.52° E) and Cuttack (20.28° N and 85.54° E), Orissa, India during January 2004 to December 2006. The testes lobules were taken as the materials for study. The animals were injected with chemicals for 6,12 and 24 hours interval after which specimens were sacrificed for collecting testes lobules. Parallel controlled sets were also prepared by injecting the solvent, distilled water to the specimens. Each compound with 0.04ml, 0.08ml and 0.125ml dose was injected separately. For each concentration of dose, the treated animals were kept for 6, 12 and 24 hours of duration to study their effect on germinal cells. Routined cytological preparations were made for both controlled and treated sets. Cells and chromosomes were observed, analysed and abnormal cells were photomicrographed. The entire data were statistically analysed and the 'Z' value was calculated from the proportions of abnormal cells of controlled set and treated sets.

RESULTS

From the normal squash preparation, a good number of metaphase plates were observed. The chromosomes have been observed to be of acrocentric in nature. In the control set, the percentage of abnormal cells having chromosomal aberrations such as stickiness, clumping, breaks, stretching and woolly appearance are found to be 1.5, 2 and 2.5; proportions 0.015, 0.02 and 0.025 for 6, 12 and 24 hours respectively (Table 1).

In treated sets, the abnormalities encountered during our observation were woolly appearance (Fig.1), corrosive effect on chromosomes (Fig.2), stickiness (Fig.3) clumping, chromatid breaks (Fig.4), gap (Fig.5), fragments, chromatin stretching (Fig.6) and bridges of chromosomes (Fig. 6) Treatment of 0.04ml dose of Gentamicin for 6, 12 and 24 hours of duration showed the percentage of abnormal cells to be 6.25, 7.25, and 8.25; proportions 0.0625, 0.0725 and 0.0825 and calculated Z value 3.653, 3.75 and 3.833 respectively. Similarly, for 0.08ml dose, percentage of abnormal cells was observed to be 6.75, 7.75, and 9.5; proportions 0.0675, 0.0775, and 0.095; and Z value 3.75, 3.833 and 4.375 respectively. Percentage of abnormal cells in 0.125ml dose was calculated to be 7.25,8.5 and 9.75; proportions 0.0725, 0.085, 0.0975 whereas the Z value 3.993, 4.276 and 4.531 respectively. From this observation, it is evident that the percentage of abnormal cells was more with the increase of dose and duration of exposure of Gentamicin (Table 2).

Treatment of 0.04 ml dose of Tobramycin for 6, 12, and 24 hours showed the percentage of abnormal cells as 5.25, 6.5 and 7.5; proportions 0.0525, 0.065 and 0.075 and calculated Z values 3.125, 3.214, and 3.333 respectively. Similarly, the treatment of 0.08ml dose for respective three hours, the percentage of abnormal cells was found to be 6.25, 7.5 and 8; proportions 0.0625, 0.075, 0.08 and Z value 3.653, 3.666 and 3.666, respectively. In the treatment of 0.125ml of Tobramycin for 6, 12 and 24 hours the percentage of abnormal cells was found to be 6.75, 7.75, and 8.75; proportions 0.0675, 0.077 and 0.0875 and Z value 3.75, 3.8 and 3.906, respectively. The percentage of abnormal cells increases with the increasing dose and duration of Tobramycin (Table 3). The calculated values (Tables 2 and 3) of Z for both the drugs were greater than Z value at 1 per cent confidence level (The tabulated value at 1 per cent is 2.58). So, these drugs have mutagenic effect on the germinal cells of insects.

DISCUSSION

Auerbach and Robson (1944) first demonstrated the chromosomal aberration by chemical mutagen. Effects of organic pesticides on mammalian cells have been reported by Somers and Hu 1962; Palmer et al., 1972; Bhunya and Behura, 1987; Pati and Bhunya, 1987 and Spencer et al., 1994. Kost (1949), Sobes (1956), Smith (1966), Mc Laughlin et al., (1969), Mahr and Miltenburger (1976), Basak and Konar (1977) and Pandit (1986) have reported genotoxic potential of different mutagens and chromosomal aberrations such as stretching, breakages, clumping, bridges and extreme fragmentation. The chemically induced chromosomal aberration in insects has been reported by several workers (Klassen et al., 1969; Abdel-Hameded et al., 1970; Manna and Parida, 1972; Vogel and Chandler 1974; Bhunya and Das 1976; Zimmering et al., 1985; Bhunya and Behura, 1986; Behera and Bhunya, 1987 and Mohanty et al.,2006.

In this study, both Gentamicin and Tobramycin are found to be clastogenic and mitotoxic to genetic material of grasshopper. The percentage of abnormal cells increased with increased concentration of dose and duration of the treatment. From the statistical analysis, it is inferred that both the drugs have significant mutagenic effect on genetic material of grasshopper. Therefore, nonuse or quite restricted use of these drugs is suggested to maintain the genetic stability and sustain genetic make up of human population.

Table 1. Percentage and proportion of abnormal cells in control sereis [Solvent: Distilled water]

SI No	Duration of Injection (in hrs)	Types of abnormalities						Total no. of abnormal cells	Percetage of abnormal cells	Proportion of abnormal cells
		Total no. of cells counted	Stickiness	Clumping	Severe breaks	Stretching	Woolly appearance			
1.	6	400	2	-	1	1	2	6	1.5	0.015
2.	12	400	2	2	1	1	2	8	2	0.02
3.	24	400	2	4	1	-	3	10	2.5	0.025

Table 2. Percentage of proportion of abnormal cells found in different doses and duration of treatment of Gentamicin

SI No	Dose of treatment (ml)	Duration of treatment (in hours)	Total No. of cells counted	Types of abnormalities									Total No. of abnormal cells	Percentage of abnormal cells	Proportion of abnormal cells	Z Value
				Severe breaks	Woolly appearance	Stickiness	Clumping	Breaks	Stretching	Gaps	Laggard	Bridges				
1		6	400	10	4	4	7	-	-	-	-	-	25	6.25	0.0625	3.653*
	0.04	12	400	11	5	4	8	1	-	-	-	-	29	7.25	0.0725	3.75*
		24	400	12	5	2	10	2	2	-	-	-	33	8.25	0.0825	3.833*
2		6	400	10	4	4	7	1	1	-	-	-	27	6.75	0.0675	3.75*
	0.08	12	400	10	4	3	8	2	2	1	1	-	31	7.75	0.0775	3.833*
		24	400	10	2	6	6	5	5	1	1	2	38	9.5	0.095	4.375*
3		6	400	8	4	5	3	2	2	1	1	3	29	7.25	0.0725	3.993*
	0.125	12	400	10	4	6	3	3	2	1	3	2	34	8.5	0.085	4.276*
		24	400	8	7	5	4	4	3	3	4	1	39	9.75	0.975	4.531*

* Significance at 1% level

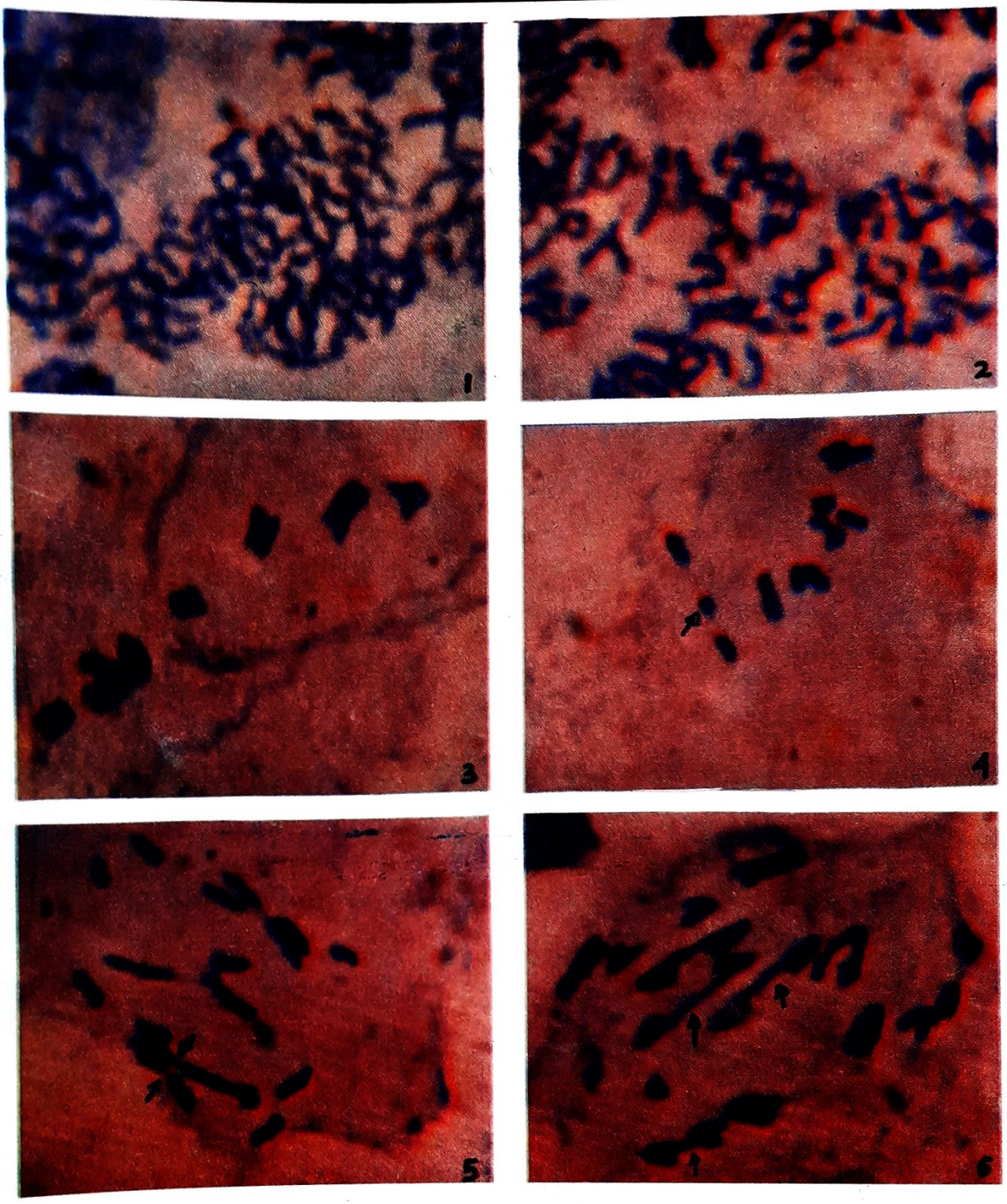
The tabulated value at 1% is 2.58

Table 3. Percentage of proportion of abnormal cells found in different doses and duration of treatment of Tobramycin

Sl No	Dose of treatment (ml)	Duration of treatment (in hours)	Total No. of cells counted	Types of abnormalities									Total No. of abnormal cells	Percentage of abnormal cells	Proportion of abnormal cells	Z Value
				Severe breaks	Woolly appearance	Stickiness	Clumping	Breaks	Stretching	Gaps	Laggard	Bridges				
1	0.04	6	400	10	3	2	4	1	1	-	-	-	21	5.25	0.0525	3.125*
		12	400	10	4	2	6	1	2	-	1	-	26	6.5	0.065	3.214*
		24	400	12	5	3	6	2	1	-	1	-	30	7.5	0.075	3.333*
2	0.08	6	400	8	6	3	4	-	1	1	2	-	25	6.25	0.0625	3.653*
		12	400	10	4	4	6	1	1	-	2	2	30	7.5	0.075	3.666*
		24	400	8	6	6	8	2	-	-	2	-	32	8	0.08	3.666*
3	0.125	6	400	13	4	2	3	2	4	1	-	-	27	6.75	0.0675	3.75*
		12	400	7	7	4	5	3	2	2	-	1	31	7.75	0.077	3.8*
		24	400	12	8	5	2	3	2	-	2	1	35	8.75	0.0875	3.906*

* Significance at 1% level

The tabulated value at 1% is 2.58



Figs.1-6. (1) Woolly appearance at leptotene; (2) Corrosive effect on the chromosomes at diplotene; (3) Sticking of chromosomes at metaphase-I; (4) Chromatid break at anaphase-I; (5) Gap in chromosome at anaphase-II; (6) Chromatin stretching and chromatid bridge at anaphase-II.

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