PUNJAB UNIVERSITY

JOURNAL OF ZOOLOGY

(FORMERLY BULLETIN, DEPARTMENT OF ZOOLOGY)
UNIVERSITY OF THE PUNJAB (NEW SERIES)

Volume 12 1997

CONTENTS

Punjab Univ. J. Zool., Vol. 12, pp. 1-13, 1997

SOME METABOLIC ALTERATIONS INDUCED BY HIGH DOSES OF UNILOCULAR FILTERED HYDATID CYST FLUID ON RABBIT LIVER

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Abstract: Out of 46 rabbits acclimatized for 2 weeks to the optimal conditions of animal house, 23 were inoculated upto 7 weeks with different doses (0.50, 0.75, 1.0, 1.25, 1.50, 1.50, 1.50 ml) of filtered hydatid cyst fluid (FHCF) of sheep origin. Each dose was daily inoculated for one week and then calculated according to their body weight. Control rabbits were given distilled water with similar protocol. Three treated and three control rabbits were weekly slaughtered and biochemical analyses of liver tissue were made. It was noted that body weight increased gradually in both control and experimental rabbits but when both groups were compared, a significant reduction was noted for all the rabbits treated with FHCF except the last three doses which were kept constant. Their respective specific growth rate also showed increasing trend but with lot of fluctuations. In the liver tissues of treated rabbits increase in total protein, soluble protein ($P<0.001$), glutamate oxaloacetate transaminase ($P<0.001$), glutamate pyruvate transaminase ($P<0.01$), bilirubin ($P<0.05$), alkaline phosphatase ($P<0.001$), acid phosphatase, ribonucleic acid, deoxyribonucleic acid and free amino acid was found statistically significant when compared with their respective controls, while cholesterol level decreased in all the treated rabbits.

Key words: *Echinococcus granulosus*, rabbit, liver tissue, biochemical analyses.

INTRODUCTION

Hydatidosis or echinococcosis is of zoonotic importance, infecting not only man but also a wide variety of domestic and wild animal. It is characterized by the formation of cysts of variable size in various body organs (Thompson, 1986). The casual agent *Echinococcus granulosus* is a cestode belonging to the family Taeniidae and genus *Echinococcus* (Rudolphi, 1801). Man gets infection by ingesting *E. granulosus* eggs in food contaminated with dog faeces or from hands contaminated while handling the infected dogs (Cheesbrough, 1987).

Hydatidosis has been considered as the most important threat to the public health (Schantz, 1982; Feng and Zho, 1986). Its economic effects also arise from public health aspects, hospitalization, associated with control programmes, losses from condemnaton of affected organs from live stock at slaughters and affects animal reproduction and meat quality (Schawabe, 1986; Iqbal *et al.*, 1989).

0079-8045/97/0001-0001 $ 03.00/0

In Pakistan, livestock is constantly exposed to this disease because the poor hygienic conditions and climate are best suited for its growth and completion of life cycle. Although pathology and symptoms are generally not very dramatic hence it has given less importance. Most of the work reported from Pakistan is not only fragmentary but are also theoretical revision of already known facts about its transmission, epidemiology and prevalence (Anwar and Munir, 1980; Khan, 1982; Bilquees, 1984; Islam, 1985; Pal and Jamil, 1986; Hussain, 1987; Iqbal *et al.*, 1989).

At present no quantitative data exist for the biochemical alterations produced in naturally or experimentally infected animals. So the main objective of the present investigation is to determine the level of different biochemical alterations in the liver of rabbits parenterally administered with high doses of filtered hydatid cyst fluid of sheep origin. For this purpose, rabbits as model are given consideration because they are common in those localities where sheep, goat and cattle are raised and they can be easily infected parenterally by injecting scolex (Cameron and Webster, 1959). They are also reported susceptible to secondary echinococcosis of sheep origin (Sweatman and William, 1963) hence we used hydatid cyst fluid of sheep origin.

MATERIALS AND METHODS

Experimental animals and their maintenance

Forty six adult healthy rabbits (*Oryctologus cuniculus*) were acclimatized for 2 weeks prior to experimentation in the Animal House of the Zoology Department, Punjab University, Quaid-e-Azam Campus, Lahore. They were supplied with green fodder and tapwater *ad libitum* along with few crystals of $KMNO_4$. Whenever, required, Gentamicin injections were given to save them from different infections. Electric heater were used to maintain the room temperature at $25 \pm 2°C$. Rabbits were weighed weekly.

Collection of hydatid cyst fluid

With the help of B.D. syringe and veterinary needle (18 G), hydatid cyst fluid was aspirated from the cysts located in liver, lungs and spleen of sheeps in the local slaughter house. The fluid was transferred into sterile vials and was placed in the ice boxes containing water at 4°C. It was brought to the laboratory and filtered through Whatman's filter paper and stored in the deep freezer at -20°C for further use.

Dose administration

For inoculation, hair from the left ear's vein were removed with the help of hair removing cream and different doses (0.5, 0.75, 1.0, 1.25, 1.5, 1.5, 1.5 ml) of filtered hydatid cyst fluid (FHCF) were inoculated upto 7.0 weeks. Each dose was daily administered upto one week and then calculated according to their body weight noted in the end of each week. Control rabbits were given distilled water with similar protocol.

Three treated and three control rabbits were slaughtered weekly and their liver was

processed for biochemical analyses.

Tissue processing for biochemical analysis

Saline extract was prepared by homogenizing weighed piece of liver tissue in 6.0 ml of 0.98% NaCl solution in a motor driven high speed tissue homogenizer at 4°C. The homogenate was centrifuged at 35000 for 15 minutes and the clear supernatant was further used for the estimations.

For the estimation of some enzyme activities *i.e.*, alkaline phosphatase (AP) and acid phosphatase (AcP) methods recommended by Deutsche Graseleschaft fur Klinische Chemie (1972) modified from Bessey *et al.* (1946) was followed. Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) was estimated according to Reitman and Frankel (1957). The estimation of bilirubin and cholesterol was made according to Jendrassik and Grof (1938) and Richmond (1973). Total proteins and soluble proteins were estimated according to Biuret method (Henry *et al.*, 1974, modified by Gowenlock *et al.*, 1988). All these estimations were made by using diagnostic kits of Randox (U.K.).

Some tissue metabolites like total free amino acids (FAA) were measured according to Goodwin (1968). Nucleic acids (DNA and RNA) were extracted as mentioned by Shakoori and Ahmad (1973) and estimated according to Schneider (1957).

Statistical analysis

All the data was analysed by Student 't' test (Steel and Torri, 1981) and specific growth rate was calculated according to Odum (1971).

RESULTS

Body weight changes and some biochemical alterations induced by different doses of filtered hydatid cyst fluid of sheep origin are mentioned in Figs.1 & 2 as mean ± S.D. and percent variations from control groups.

Figure 1 shows slow but gradual increase in the body weight (mg) of both control and treated rabbits. When the treated rabbits were compared with their respective control groups they showed reduced body weight (-2.42%, -6.70%, -1.49% and -1.05%) for 0.50, 0.75, 1.0 and 1.25 ml of FHCF. However, when the dose was kept constant a significant increase (5.48%, 14.46%, 17.03%) was noted. In the control groups specific growth rate (SGR) per week increased with varied values (1.13%, 1.09%, 1.77%, 1.4%, 1.38%, 3.58%) while in experimental rabbits, SGR showed a decrease in 0.75ml dose only. Other treatments showed an increase with varied values.

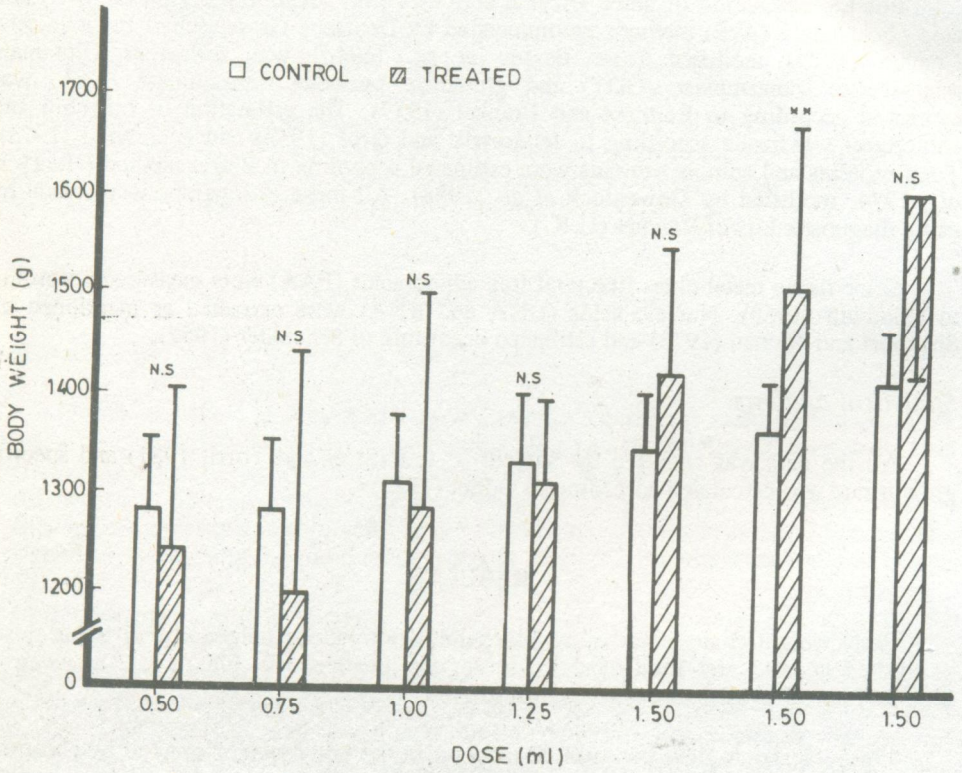

Fig. 1: **Live body weight changes in rabbits due to parenteral administration of high doses of filtered hydatid cyst fluid (FHCF). The statistical significance has been determined by Student's 't' test and the probability represented by stars *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$.**

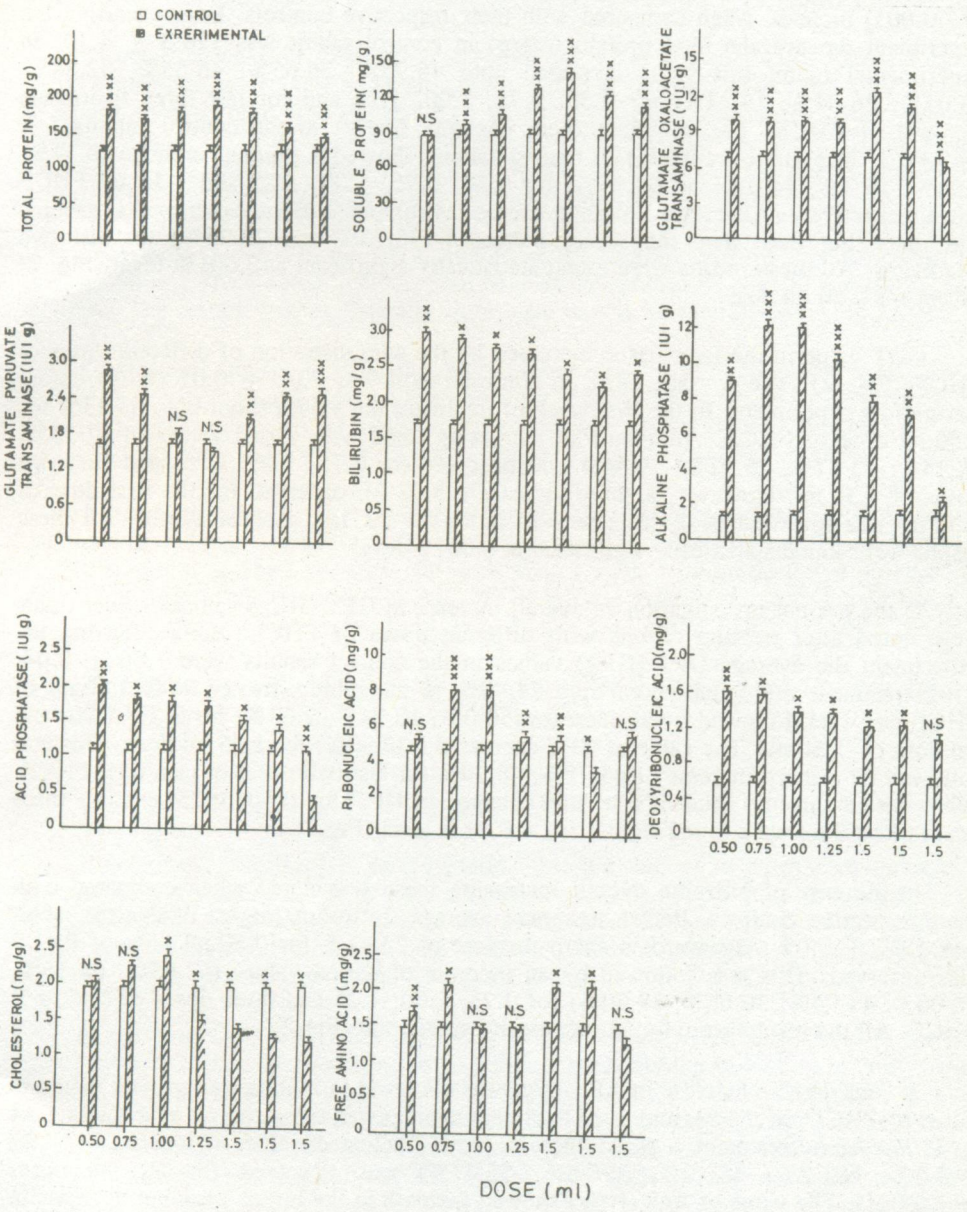

Fig. 2: **Some biochemical changes in liver of rabbit due to high doses of filtered hydatid cyst fluid (FHCF). The statistical significance has been determined by Student's 't' test and the probability represented by stars *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$.**

Total protein contents (mg/g) in the liver tissue of rabbits showed a significant ($P<0.001$) increase when compared with their respective controls. Before starting the experiment the average total protein (mg/g) in control rabbit was 126.3 ± 4.10. In experimental rabbit this value increased upto 46.95%, 36.65%, 46.55%, 52.88%, 43.07%, 26.84%, 16.23%, for 0.50, 0.75, 1.00, 1.25 and for last three treatments (1.5 ml) of FHCF. The soluble protein contents (mg/g) in the control rabbits was 89.3±0.1. The similar contents in treated rabbits showed a gradual increase (0.11%, 9.96%, 19.3%, 44.0% and 59.35%) for 0.50, 0.75, 1.00, 1.25 and 1.50 ml FHCF doses respectively. Afterward a slight decrease (as compared to the experimental groups) was noted but even then the values increased (36.73% and 25.75%) for last two treatments. All these results were found statistically significant at 0.001% level (Fig. 2) except for 0.50 ml dose.

GOT value in the liver tissue increased by the administration of different doses of FHCF (Fig. 2). The average GOT in control rabbit was 7.02 ± 0.01 (IU/g) before starting the experiment. In the first week of treatment the values shoot upto 53.13% for 0.50 ml dose of FHCF. Afterward an increasing trend with varied values of 47.15%, 48.14%, 43.87%, 75.92%, 59.54% was observed for 0.75, 1.00, 1.25, and first two doses of 1.50 ml. However, a sharp increase (75.92%), observed for the first dose of 1.50 ml was followed by a decrease (-14.24%) for the last dose of FHCF. All these results were found statistically significant at 0.001% level.

In the present investigation an overall increase in GPT (IU/g) values of liver tissue were noted after treating rabbits with different doses of FHCF. Before starting the experiment the average GPT (IU/g) values in the control rabbits were 1.66 ± 0.04. After treatment this value shoot upto 74.49% in the rabbits treated with 0.50 ml of FHCF per week followed by an increase (50.0%, 10.24%, 9.63%) for 0.75, 1.00, and 1st dose of 1.50 ml. The value of GPT decreased (-25.90%) for 1.25 ml dose. This was followed by a sharp increase of 45.78%, 48.79% for last two doses of (1.5 ml), FHCF. When tested through Student's 't' test changes in GPT for 0.50, 0.75 and last three doses of 1.50 ml were found significant at 0.01% level (Fig.2).

In the case of bilirubin overall increasing trend was noted when compared with their respective controls. Before treatment average, bilirubin (mg/g) of control rabbit was 1.73 ± 0.05. Afterwards a sharp increase of 73.41% for 0.50 ml dose of FHCF was observed. This was followed by an increase of varied values (67.63%, 64.16%, 56.06%, 41.04%, 30.05%, 39.30%) for 0.75, 1.00, 1.25 and three doses of (1.50 ml) FHCF. All the results were found statistically significant (Fig.2)

A considerable increase in AP (IU/g) was observed in rabbits treated with different doses of FHCF. In the beginning of treatment average value for control rabbit was 1.44 ± 0.01. After treatment a sharp increase with fluctuated values (526.3%, 747.2%, 740.2%, 601.3%, 452.0%, 396.5%, 53.47%) was observed for all the doses ($P<0.001$). The value of AcP (IU/g) showed increase in the initial stage but decrease in last stage. In the beginning the average AcP (IU/g) value of control rabbit was 1.15 ± 0.01. Afterward an increase with gradually decreasing values (78.26%, 60.86%, 57.39%, 53.04%, 34.78%, 25.21%) was observed for 0.50, 0.75, 1.00, 1.25 and Ist two doses of 1.50 ml of FHCF. This was again followed by a sudden decrease of

-64.34% for last dose of 1.50 ml of FHCF. The results were found statistically significant when analysed by Student 't' test (Fig.1).

In the first week of treatment the values for RNA contents were found closer to their respective controls (Fig.2) alongwith 15.33% increase. Later on this increase gradually reached upto 70.79% and 70.37% for 0.75 and 1.0 ml doses. Afterwards the process of increase in the RNA contents slowed down through 56.72, 52.10, 28.15 and 16.38% for 1.25 and last three doses of 1.50 ml of FHCF. All the results were found statistically significant except that of 0.50 ml and last dose (1.50 ml).

Maximum increase in the DNA contents was observed after the inoculation of first dose (0.50 ml). This value later on gradually declined upto 83.8% till the last dose of 1.50 ml. The results were statistically significant except for the last dose (Fig.2).

Cholesterol (mg/g) in control rabbits before starting the experiment was 2.04 $\pm$ 0.02. After treatment this value increased upto 5.88%, 12.74% and 20.09% for 0.50, 0.75, and 1.00 ml doses of FHCF. Then decrease of -24.01%, -29.90%, -37.7%, -38.23% was observed for 1.25 ml and last three doses of 1.50 ml ($P<0.05$).

In the beginning the average values for plasma free amino acids (mg/g) in control rabbits was 1.53 $\pm$ 0.05. After significant increase (13.7% and 42.48%) for 0.50 and 0.75 ml a slight decrease (-0.65% and -1.30%) for 1.00, 1.25 ml dose was observed. In the end a significant increase (39.21%, 42.48%) was noted for the first two doses of 1.50 ml followed by a decrease of -14.37% for the last dose of FHCF (Fig.2).

The results were found statistically significant for 0.50, 0.75 and first two doses of 1.50 ml (Fig.2).

DISCUSSION

Biochemical effects of filtered hydatid cyst fluid on rabbit liver were statistically significant when compared with their respective controls. The results showed that high doses of FHCF have not only altered the biochemical parameters but also the general appearance of rabbits that became pale and weak with the passage of time. They further showed loss of appetite, agility and shedding of hair.

In the present investigation live body weight showed decreasing trend in the first four stages, and then increased in the last three stages. Similar reduction in body weight has also been reported by Pandey (1971) who studied the effect of hydatidosis in liver, lungs and spleen of goats in Patna (India) and reported that gross changes in these tissues were due to fluid which seeped out of the hydatid cyst and damage the surrounding cells thereby causing condemnation of the organs that leads to the reduction in weight. Economic losses due to hydatidosis through low quality and reduced yield of milk, meat and retarded growth have also been reported on the same basis by Anonymous (1985), Schwabe (1986) and Iqbal *et al*. (1989).

It has been an established fact that decrease in body weight is associated with liver enlargement which may be either due to accumulation of triglycerides in the liver (Kohli *et al*., 1975) or due to increased energy requirements for the induction of body's defence mechanism to detoxify that chemical (Zhou *et al*., 1985). However, in the last three doses, the increase in the body weight can be attributed to some resistance which the animals have attained against filtered hydatid cyst fluid (FHCF).

Liver is not only the primary site for biotransformation of foreign compounds but also the centre of drug metabolism which makes it greatly vulnerable to toxic substances. The role of liver in metabolic conversion of foreign compound is even more important in its susceptibility to biochemical injury (Zimmerman, 1974).

Since, HCF contained different kind of enzymes that are liable to alter the hepatic physiology and its metabolites. In most of the cases these enzymes leaked out from the necrotic hepatocytes into the blood stream in abnormal amounts. Several of these soluble enzymes have been considered as indicators of liver function and damage (Kulkarni and Hodgson, 1980).

Frayha and Haddad (1980) noted the presence of protein contents, enzymatic protein (glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, phosphates) and dehydrogenases in HCF obtained from different intermediate host. It has earlier been reported that out of ninteen protein components isolated from HCF, 10 were antigens of parasitic origin (Biguet *et al*., 1962; Capron *et al*., 1962; Chordi and Kagan 1965; Castagnari and Pozzuoli, 1969). Host immunoglobulins have also been reported in the cyst wall, fluid and on the surface of protoscoleces (Kassis and Tanner, 1977). *In vitro*, studies have also proved that hydatid fluid of the cyst took proteins from their surroundings (Coltortic and Varela-Dias, 1975; Hustead and William, 1977). Although proteins are large molecules and their size have biological significance which prevents them from leaking out of cells through the plasma membrane (Conn and Stumpt, 1976). In the present work an over-all increase in the total and soluble proteins was estimated in the liver tissue of rabbits. This was probably due to the fact that protein synthesis was increased in the liver to over come the toxic effects of FHCF or utilization of free amino acid contents for energy needs under stress conditions, or in the absence of glucose oxidation (Abdel Salam *et al*., 1982).

Transaminases are enzymes involved in the metabolism of amino acids and are responsible for conversion of amino acids to α-Ketoacids which later on enter into Krebs cycle and are metabolized to produce energy for different metabolic processes (Meena *et al*., 1978).

The present investigation showed an increasing trend of GOT in the liver tissues of experimental rabbits. In the start this increase may be due to the presence of GOT in FHCF that was inoculated to the experimental rabbits (Frayha and Haddad, 1980) and this may be further attributed to the pathological response of the hepatocytes of liver (Sanchez and Sanchez, 1971). Moreover, a decrease in hepatic GOT in the 7th week may be due to the blockage at transcription or translation level (Meena *et al*., 1978) or some disfunctioning of liver (Ramaligam and Reddy, 1981) or cell damage (Hendrickson and Bowden, 1976; Meany and Pocker, 1979) or development of some resistance against

the incoming toxins present in the FHCF.

Tissue GPT activity is generally required for transamination purpose. In the present investigation the rise in the activity of this enzyme is an indication of disturbed liver function, necrosis of hepatic cells which favour cellular damage or gluconeogenesis through which amino acids may be transmitted and utilized for energy requirements or due to efficient conversion of alanine to pyruvate which enters into TCA cycle to compensate for energy requirements. Another important reason for increased hepatic GPT is that some GPT was already present in that FHCF which was administered to the rabbits (Frayha and Haddad, 1980).

In the present investigation the increased bilirubin contents may be attributed to some bilirubin already present in HCF (Frayha and Haddad, 1980) that was given to the rabbits, and increased break down of haemoglobin (hemolysis). About 85% of bilirubin is formed from haemoglobin liberated from senescent erythrocytes destroyed by reticulo endothelial cells in the spleen. The remaining 15% is derived from other hemoproteins which are mainly cytochromes (Benjamin, 1985).

Alkaline phosphatase is a membrane bound enzyme found at the bile pole of hepatocytes, pinocytic vesicles and golgi complex. It catalyzes transphosphorylation reactions and involved in the hydrolysis of phosphate monomers at alkaline pH (9.0). They play important role in the transport of sugar and phosphate in liver and other tissues (Benjamin, 1985). In the present investigation sharp increase in the AP activity at different doses of FHCF was probably due to increased active transport process for supply of nutrients that can be used in energy generation to counter the toxic effects of FHCF.

Acid phosphatase (AcP) is a hydrolytic enzyme found in the lysosomal fraction of the cell (deDuve, 1955). There are also extralysosomal acid phosphatases which are found in the endoplasmic reticulum and cytoplasm and used to estimate the interference with catabolic and autophagic processes in the liver. In the present investigation its increased activity may be attributed to the increased breakdown activities of waste cellular components produced as a result of toxic effects of FHCF. Another reason for increased AcP activity may be due to increased biosynthesis of enzyme protein to fulfill the elevated demand in the cell. Thus, it can be safely suggested that in the present investigation AP and AcP activities increased to meet the stress condition produced by incoming FHCF.

In this study it was further investigated that among the nucleic acids, RNA contents gradually increased but later on decreased with the increase of dose and time. While DNA showed an overall increasing trend for all the doses of FHCF. This can be explained that under stress condition the DNA level increased to produce large amount of RNA which then further take part in protein synthesis (this is also confirmed by increased protein contents in the present investigation). Increased level of DNA is also due to the presence of DNA in the incoming HCF (McManus and Smyth, 1982). However, inhibitory effects of FHCF on RNA biosynthesis is another important consideration. It is also important to note that under stress condition the animal minimize the food uptake and due to decrease in dietry proteins and energy, RNA in the

liver cells also decreased (Waterlow and Hauge, 1978).

In the present study cholesterol content initially increased and latter on decreased. As cholesterol is precursor of steroid hormones so possibly it is used in the synthesis also. In the present investigation its elevated level for 0.50, 0.75, 1.00 ml doses indicates that it is not being used in the biosynthesis of steroid hormone. Afterward a decrease in cholesterol content was observed for 1.25 ml and last three doses (1.5 ml) of FHCF. This can be explained that under stress condition the animal minimize the food uptake and under starved conditions the cholesterol is metabolized to meet the energy requirements of the animals. Another reason for decreased level of cholesterol in liver is that incoming HCF has inhibitory effect on cholesterol synthesis. Under disease condition the liver cholesterol ester decrease due to decreased quantity of liver enzyme that influence esterification (Benjamin, 1985). In the present study the decreased cholesterol level in the treated rabbits can be attributed to the liver damage due to inhibitory effect of FHCF.

Among other biochemical components free amino acids fluctuated a lot. Amino acids are used in protein synthesis. Their increase in the initial three stages showed that during this period glucose was not available for energy generation, rather amino acids, available in surplus/higher amounts were being utilized for various activities of liver tissue. Another reason for increased FAA may be due to the release of amino acids from liver, as a result of proteolysis in the liver tissue. Consequently the restoration of plasma free amino acid contents to initial pre-treatment level was also due to their utilization in gluconeogenesis (Mommsen, 1986).

From the results of present study it can be concluded that different doses of FHCF injected, parenterally into rabbits were high enough to induce significant alterations in the liver enzymes and other metabolites.

Acknowledgements

Financial assistance provided by Pakistan Science Foundation (P.U. Agr. 137) is gratefully acknowledged.

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(*Received January 25, 1998*)

Punjab Univ. J. Zool., Vol. 12, pp. 15-23, 1997

EFFECT OF TRANSPLANTATION ON ENTIRE ANTERIOR TIBIALIS MUSCLE OF RABBIT, *ORYCTOLAGUS CUNICULUS**

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Abstract: Effect of transplantation on entire anterior tibialis muscle (ATM) has been studied in rabbits. It was observed that there was an initial phase of degeneration of almost all the original muscle fibres followed by a regeneration of new myotubes within 10 days of transplantation. The process of regeneration was slow in this muscle. All the morphometric parameters studied were decreased till the end of experiment. The DNA and RNA contents showed increase while protein contents decreased during whole period.

Key words: Regeneration, morphometric studies, nucleic acids, muscle DNA, muscle RNA, total proteins.

INTRODUCTION

The transplantation of entire mammalian skeletal muscles has been accomplished in both the research laboratory (Carlson *et al.*, 1978; Sloper and Partridge, 1980; Albani and Vrbova, 1985; Martin *et al.*, 1990; Gill and Shakoori, 1995, 1996) and clinical practice (Thompson, 1971, 1974; Hakelius, 1974; Henriksson *et al.*, 1985). The degeneration and regeneration process proceeds after transplantation so that the graft regains its structural and functional characteristics (Faulkner *et al.*, 1980; Thomas *et al.*, 1984). The size of the muscle in relation to total body size of the animal is of considerable importance in terms of its ability to undergo regeneration in a transplanted condition. Many authors reported good regenerative ability of the anterior tibialis muscle (ATM) (Salafsky *et al.*, 1974; Sadeh *et al.*, 1985). The present paper describes the regenerative ability of ATM in rabbit, *Oryctolagus cuniculus*.

MATERIALS AND METHODS

The male rabbits, *Oryctolagus cuniculus* of 1.00-1.30 kg wieght were used in this

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*Part of Ph.D. thesis of first author, submitted to University of the Punjab, Lahore, Pakistan.

experiment. The animals were acclimatized for two weeks in a separate room of the Animal House of the Department of Zoology, University of the Punjab, under semi-controlled temperature conditions. The animals were provided with fresh green fodder (clover) and tap-water.

Transplantation

The animals were operated upon under thiopentone sodium (50 mg/kg body weight) anaesthesia. The procedure for transplantation of ATM was same as described by Gill and Shakoori (1996). For control, both right and left legs were sham-operated and muscles were left intact. After various time intervals *viz.*, 1, 3, 5, 7, 10, 15, 30 and 60 days, the muscles were removed and processed for histological, morphometric and biochemical studies.

Morphological and histological studies

For morphological studies both right and left legs were dissected, muscles were exposed, their proximal and distal connections were snipped, weighed, breadth, length and colour was noted. For histological studies, muscles were fixed, processed and sectioned at 6 μm.

Morphometric studies

Following parameters were considered for morphometric studies: (*i*) total muscle area, determined by planimetry using Liesegang A 30S microprojector; (*ii*) number of muscle fibres / microscopic field and number of nuclei / muscle fibre was counted at a magnification of 500x and 1250x respectively; (*iii*) orthogonal diameter (major axis x minor axis) of muscle fibres, measured with the help of an ocular micrometer at a magnification of 500x and that of nuclei at 1250x.

Biochemical analysis

For extraction of nucleic acids, the method described by Shakoori and Ahmad (1973) was adopted. The estimation of DNA and RNA was done according to Schmidt and Thannhauser procedure as described by Schneider (1957). The procedure of Lowry *et al.* (1951) was adopted for estimation of protein contents.

RESULTS AND DISCUSSION

Body weight

The weight of animal showed maximum decrease of 16% during first 15 days of transplantation.

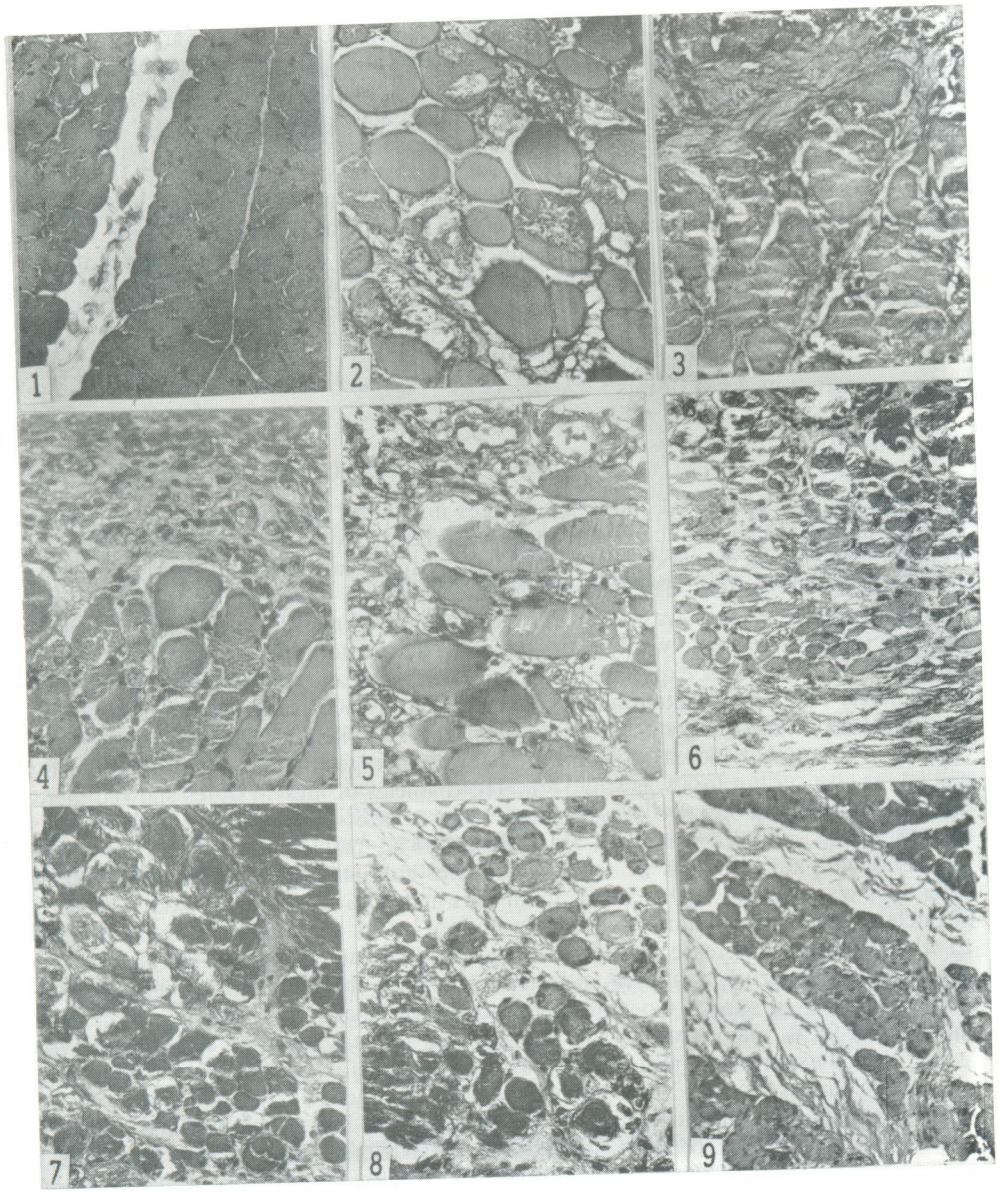
Morphological and histological studies

Figures 1-9 show the sequence of various histological changes in control and

transplanted ATM of rabbits. In control muscle, muscle fibres were enclosed in muscle bundles which were compactly arranged. The nuclei were present toward the periphery. Morphologically, the wound was completely healed after 10 days of transplantation. The colour of the regenerate was pink with yellow patches. Histologically, twenty-four hour after transplantation, majority fibres hypertrophied and a few showed early degenerative changes. Many fibres were sarcolysed with increase in connective tissue within 3 days. The degenerative changes proceeded towards the centre after one week. Following this, regeneration initiated towards the periphery in the form of myoblasts and myotubes alongwith lot of connective tissue. By day 15, regenerated myotubes were basophilic with central nuclei. During the remaining days, amount of regenerated fibres increased with much reduced diameter and central nuclei. Lot of connective tissue was still present in between the regenerated fibres. The regeneration process was slow in this muscle and seems to be due to bigger size of the muscle. Sadeh *et al.* (1985) reported that muscle structure were restored after one week. Robertson *et al.* (1993) reported limited capacity for regeneration in ATM of rats.

Morphometric studies

The Figures 10 and 11 show the changes in weight, dimensions, total muscle area, number and diameter of nuclei and muscle fibres in ATM after transplantation. The weight of muscle increased during first 3 days of transplantation and seems to be due to edema caused by surgery. The weight decreased within 5 days and continued till the end of experiment when loss was 48%. Just like weight of muscle, breadth of muscle also showed increase within twenty-four hours. The breadth decreased afterwards and continued till the end of experiment (28%). The length and total muscle area decreased immediately after transplantation and continued till day 60. The number of muscle fibres also decreased after one day and continued till day 10, when it was 56% less than that of control. The number increased with the progress of regeneration and continued till the end of experiment but loss was still 37%. The diameter of muscle fibres showed increase during first 3 days along two axes. It was decreased after degeneration and continued till day 15 when loss was maximum (minor axis 34%, major axis 38%). The diameter increased with the progress of regeneration and continued till the end of experiment when loss was reduced to 9% along minor axis and 20% along major axis. The number of nuclei / muscle fibre started increasing within 3 days and continued to do so till day 15 when maximum increase of 70% was recorded. The number decreased afterwards but at the end of experiment, it was still 32% more than that of control. The diameter of nuclei also showed similar types of results. It showed maximum increase within 15 days (minor axis 95%, major axis 61%). The diameter decreased during remaining days but at the end of experiment, minor axis was 51% and major axis 38% more than that of control. Changes in all these morphometric parameters correlate well with the histological studies. Gill and Shakoori (1996) observed loss in number and diameter of muscle fibres in ATM of rat. The reduced mean fibre diameter and fibre size was observed in minced ATM regenerate system (Neerunjun and Dubowitz, 1977; Salafsky *et al.*, 1974). The human muscle adaptation to physical demand occur by means of variation in fibre types, number of muscle fibres and fibre size over the muscle cross-section (Henriksson *et al.*, 1985).



Figs.1-9: Histological section through transplanted anterior tibialis muscle of rabbit; 1, control, 2, day 1, showing early degenerative changes; 3, day 3, note degenerated fibres with macrophage activity; 4, day 5, note replacement of muscle fibres by connective tissue; 5, day 7, showing degenerative changes without any traces of myoblasts; 6, day 10, showing early regeneration towards the periphery; 7, day 15, note regenerated fibres; 8, day 30, note regenerated fibres with mostly central nuclei; 9, day 60, note reduced diameter of regenerated fibres. Stain: Haematoxylin and Eosin; Magnification: all 100x.

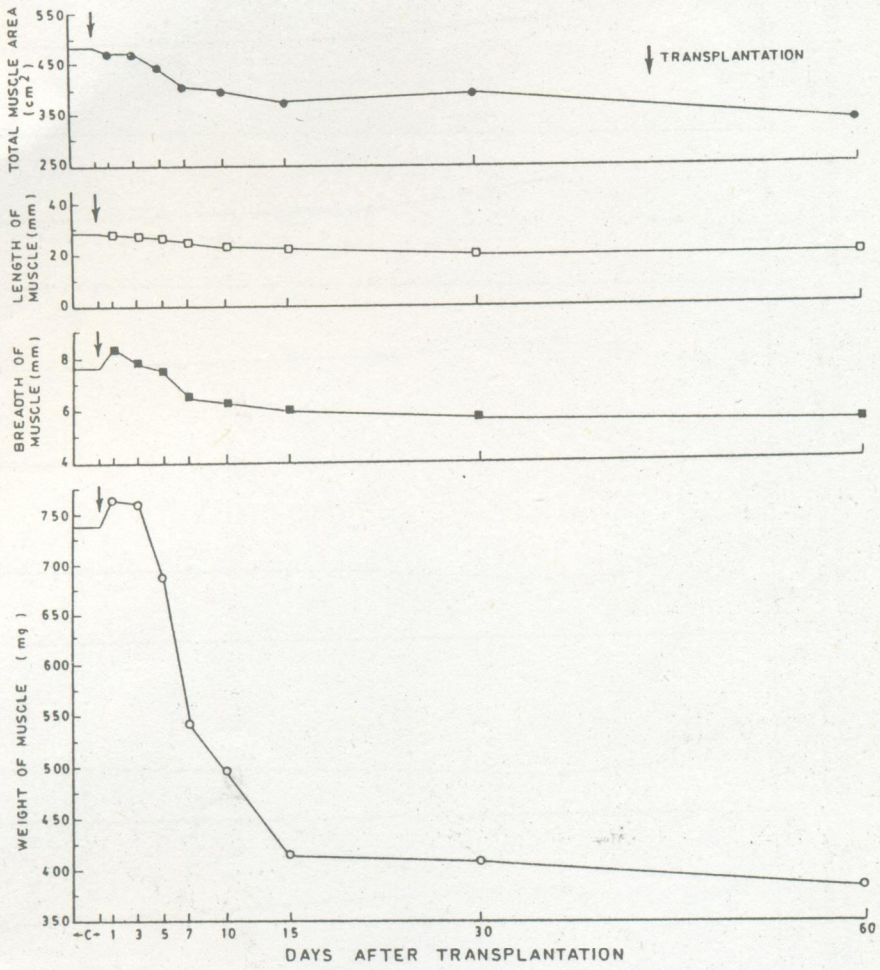


Fig. 10: **The weight of muscle, dimensions and total muscle area after transplantation of anterior tibialis muscle in rabbit.**

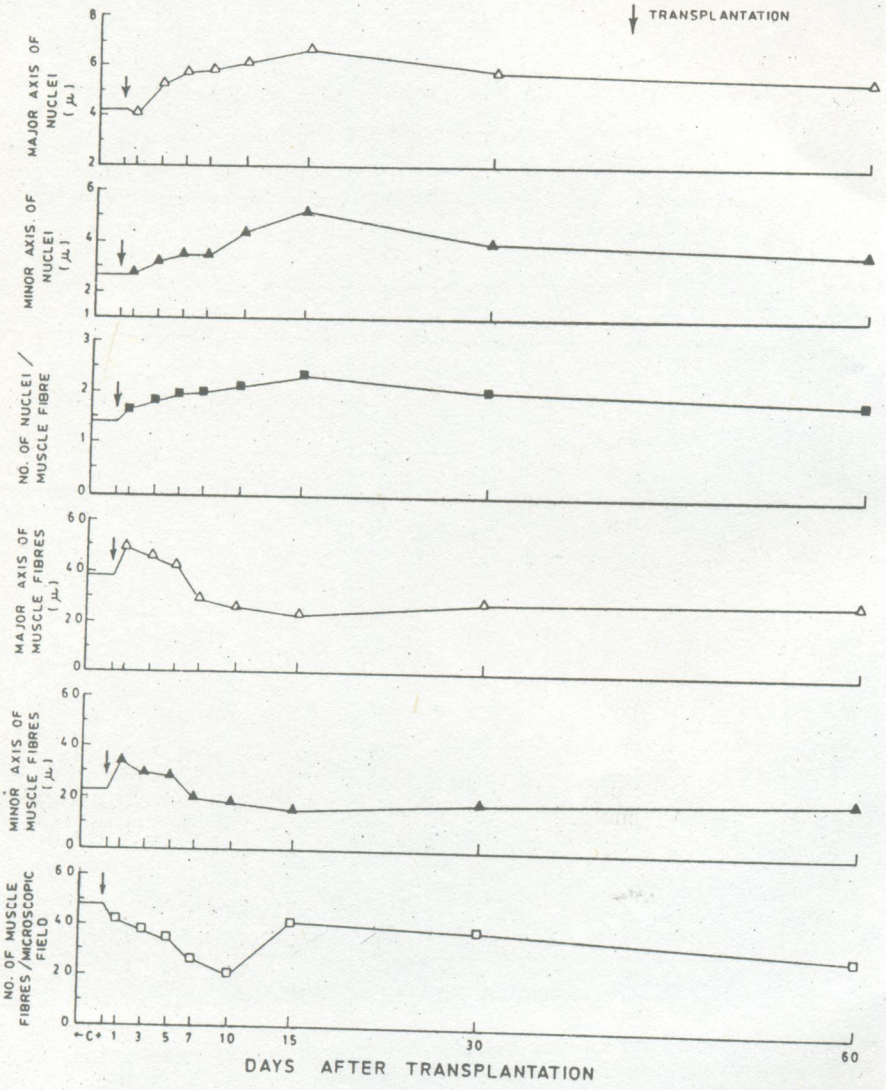


Fig. 11: **Changes in various morphometric parameters of anterior tibialis muscle of rabbit after transplantation.**

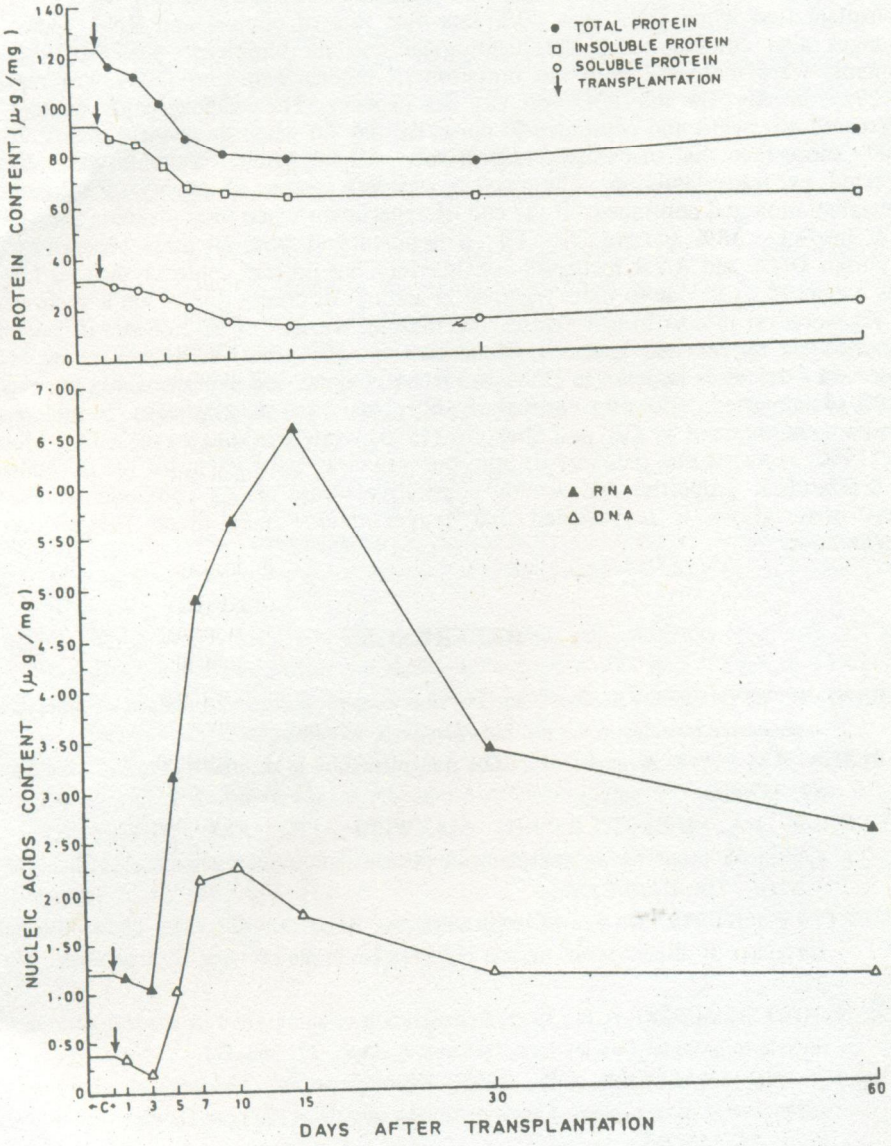


Fig. 12: **Effect of transplantation on the nucleic acids and protein content in rabbit anterior tibialis muscle.**

Nucleic acids and protein contents

Figure 12 shows changes in DNA, RNA and protein contents of control and transplanted ATM. The DNA and RNA contents decreased during first 3 days of transplantation when DNA was 50% less than that of control and RNA 14%. These changes also coincide with the histological and morphometric studies. Both these contents were increased with the initiation of regeneration and DNA was maximum (5.29x) on day 10 and RNA on day 15 (5.44x). The nucleic acids content were decreased afterward and continued to do so till day 60 when these were only 2.76x and 2.07x more than that of control, respectively. All the protein contents were adversely effected by transplantation. The protein contents decreased twenty-four hours after transplantation and continued till the end of experiment when total proteins showed 42% loss, insoluble 38% and soluble 53%. It is postulated from all these observations that although DNA and RNA increased considerably but protein contents showed loss. All this happened to be due to more increase in amount of connective tissue and slower rate of regeneration due to bigger size of the muscle. Variability in number of nuclei was obtained in regenerated muscles (Robertson *et al.*, 1993). Gallucci *et al.* (1966) observed 4-10 times increase in DNA and RNA contents and explained this increase as a result of increased cellularity particularly fibroblasts and macrophages. Similar type of results were obtained by Gill and Shakoori (1996) while working on rat ATM. Martin *et al.* (1990) reported that recovery of upto one year was insufficient for the normalization of biochemical properties and several connective tissue matrix components. From all these observations it is revealed that bigger muscle take much longer time for regeneration.

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(Received: July 30, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 25-29, 1997

ANALYSIS OF *TRIPLOPHYSA YASINENSIS* COMPLEX (PISCES : NEMACHEILIDAE)

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Abstract: *Triplophysa yasinensis* (Alcock, 1898), *T. choprai* (Hora, 1934) and *T. kashmirensis* (Hora, 1934) are closely related and show similarities in various characters. Hence, *T. yasinensis* and *T. kashmirensis* were treated as the same species. Similarly, *T. yasinensis* and *T. choprai* were merged together. However, on the basis of the statistical analysis significant differences were discovered in the three species. So these three are treated as valid species.

Key words: Taxonomy, fish classification.

INTRODUCTION

The fishes of the genus *Triplophysa* are distributed in the High Asian Ichthyogeographical Region. Among these, *Triplophysa yasinensis* (Alcock, 1898), *T. choprai* (Hora, 1934) and *T. kashmirensis* (Hora, 1934) are very closely related. On the basis of their close similarity Menon (1987) merged *T. kashmirensis* into the synonymy of *T. yasinensis*, while Mirza and Alam (1994) merged *T. choprai* into the synonymy of *T. yasinensis*. On detailed statistical analysis, it was found that the three species, although showing similarity in some characters, are nevertheless distinct in many aspects. So it seems desirable to treat them as three valid species.

MATERIALS AND METHODS

Fishes for this study were collected from different parts of Pakistan and Azad Kashmir: *T. choprai* (n=10) from Chitral, Swat and Dir (Fig.1); *T. yasinensis* (n=10) from the river Hunza near Gulmit (Fig.2) and *T. kashmirensis* (n=7) from the river Neelum near Keran, Neelum Valley, Azad Kashmir (Fig.3).

Total length, standard length, head and snout lengths, body and head height; predorsal, prepelvic and preanal distances; lengths of dorsal, pectoral, pelvic, anal and caudal fins; diameter of eye and interorbital distance; base of dorsal, pectoral and pelvic

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fins; length of maxillary, inner and outer rostral barbels and caudal peduncle; and maximum and least height of caudal peduncle were taken into account. Menon (1987) was followed for measurements and meristic trait count.

Data was subjected to statistical analysis to evaluate the level of differences (Steel and Torrie, 1981) for each parameter separately.

RESULTS

Base of dorsal fin, lengths of inner rostral barbels and caudal peduncle were found to be similar (non-significantly different) in the three species, whereas length of dorsal, pectoral and pelvic fins, total, standard, head and snout lengths, head and body height; predorsal, prepelvic, preanal and interorbital distances; diameter of eye; lengths of anal and caudal fins, maxillary and outer rostral barbels, base of pectoral and pelvic fins and maximum and minimum height of caudal peduncle were found to be significantly different ($P < 0.001$; Table 1).

DISCUSSION

Triplophysa yasinensis complex of species are composed of *T. yasinensis* (Alcock, 1898), *T. choprai* (Hora, 1934) and *T. kashmirenois* (Hora, 1934). These three species are very closely related and show overlapping of many characters. The similarity between *T. yasinensis* and *T. kashmiransis* was noted by Menon (1987). So he treated these two species as one. *T. yasinensis* and *T. choprai* also show similarities in many characters and hence were treated as the same species by Mirza and Alam (1994).

In the present study the different characters were subjected to statistical analysis. It was found that the three species differ significantly in such characters like: total length, standard length, head length, head height, body height, predorsal distance, prepelvic distance, preanal distance, diameter of eye, interorbital distance, snout length, length of dorsal fin, length of pectoral fin, length of pelvic fin, length of anal fin, length of caudal fin, base of pectoral fin, base of pelvic fin, length of outer rostral barbels, length of maxillary barbels, maximum height of caudal peduncle, least height of caudal peduncle.

So it seems desirable to treat these three species as valid. Among these, *T. yasinensis* is distributed in the upper parts of the Indus from Skardu to Tarbela and its tributaries like Gilgit, Hunza etc; *T. choprai* is restricted to the upper parts of the rivers Chitral, Panjkora and Swat, while *T. kashmirensis* is primairly distributed in Kashmir and Jammu (rivers Jhelum, Neelum and Poonch etc.). Menon's (1987) record of *T. yasinensis* from the river Tawi near Chineni is most probably, based on *T. kashmirensis*.

Acknowledgements

We are grateful to Mr. M. Rafique, PMNH, Islamabad, for presenting specimens

Table 1: Comparison of different characters of the three species

Characters	*T.yasinensis* (n = 10)	*T. choprai* (n = 10)	*T. kashmirensis* (n = 7)	LSD at P 0.05
Total length	12.41±0.29	10.99±0.55	13.44±0.42	1.29
Standard length	10.46±0.26	9.26±0.48	11.31±0.37	1.13
Head length	2.13±0.04	1.96±0.08	2.48±0.11	0.23
Head height	1.12±0.02	1.05±0.06	1.34±0.05	0.14
Body height	1.37±0.06	1.21±0.07	1.74±0.08	0.21
Predorsal distance	5.22±0.12	4.58±0.23	5.73±0.20	0.55
Prepelvic distance	5.25±0.12	4.54±0.20	5.93±0.17	0.47
Preanal distance	7.07±0.16	6.24±0.30	7.78±0.23	0.84
Diameter of eye	0.2±0.00	0.26±0.02	0.3±0.00	0.03
Interorbital distance	0.5±0.01	0.41±0.02	0.67±0.04	0.13
Snout length	1.01±0.02	0.87±0.12	1.18±0.05	0.27
Length of dorsal fin	1.94±0.05	1.84±0.08	2.2±0.09	0.23
Length of pectoral fin	1.96±0.04	1.82±0.1	2.16±0.08	0.23
Length of pelvic fin	1.66±0.04	1.58±0.08	1.93±0.09	0.22
Length of anal fin	1.66±0.04	1.58±0.08	1.91±0.08	0.20
Length of caudal fin	1.95±0.05	1.75±0.08	2.13±0.07	0.20
Base of dorsal fin	1.48±0.06	1.39±0.07	1.57±0.05	N.S.
Base of pectoral fin	0.5±0.02	0.88±0.14	0.59±0.03	0.28
Base of pelvic fin	0.4±0.00	0.37±0.03	0.5±0.02	0.06
Length of inner rostral barbels	0.47±0.02	0.39±0.02	0.34±0.03	N.S.
Length of outer rostral barbels	0.71±0.08	0.58±0.02	0.79±0.04	0.09
Length of maxillary barbels	0.7±0.21	0.51±0.23	0.86±0.02	0.06
Length of caudal peduncle	2.7±0.08	2.36±0.12	2.63±0.10	N.S.
Maximum height of caudal peduncle	0.69±0.02	0.64±0.03	0.93±0.03	0.08
Least height of caudal peduncle	0.5±0.01	0.41±0.02	0.68±0.02	0.05

N.S.: Non-significane
LSD: Least significant difference

Fig. 1: *Triplophysa choprai* from Kalam (Swat), **1" length = 5.7 cm**

Fig. 2: *Triplophysa yasinensis* from Hunza, **1" length = 6.1 cm**

Fig. 3: *Triplophysa kashmirensis* from Keran and river Neelum, **1" length = 5.4 cm**

of *Triplophysa* from Northern Areas, Chitral, Swat, Dir and Azad Kashmir on which this study is mostly based.

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(Received: July 3, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 31-34, 1997

USE OF ULTRA-VIOLET SPECTROPHOTOMETRY FOR DETERMINATION OF INSECTICIDES AND AROMATIC HYDROCARBON POLLUTANTS

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Abstract: Insecticides and other aromatic hydrocarbons are major pollutants in our environment. Their biodegradation studies involve determination of the quantity of these compounds, their residues or intermediates left over during the process. Their determinations involve high cost instruments and tedious preparations. In the present report UV absorption picture of some aromatic hydrocarbons (phenol and sodium benzoate) and insecticides (diazinon, chlorfenvinphos, fenitrothion, chlorpyriphos, methyl parathion, monocrotophos, profenophos, methomidophos and dichlorvos) was taken which showed a definite pattern of absorption ranges and the wavelength scans indicated a specific wavelength at which the absorption was maximum. These UV absorption spectra were indicated to be useful and economical for evaluation of the pesticides and determination of their biodegradation.

Key words: UV absorption spectra, organophosphates, pesticides, insecticides, aromatic hydrocarbons, wavelength scan.

INTRODUCTION

Macromolecules like DNA and proteins absorb ultraviolet (UV) radiation at 260 and 280 nm wavelengths respectively, due to the presence of aromatic rings in nucleotides of DNA and aromatic amino acids *i.e.* phenylalanine, tyrosine and tryptophan in proteins. UV absorption phenomenon has been used to estimate the genome size of various organisms, to determine the repeated DNA sequences and the amount of DNA and proteins in solutions (Britten and Kohne, 1971; Jelenek and Schmid, 1982; Cedergren, 1993). Some of the common techniques employed for the determination of organic chemicals are high performance liquid chromatography (HPLC), gas chromatography, UV spectrophotometry and particle beam mass spectrometry. UV spectrophotometry has been widely used for the determination of various types of chemicals and their derivatives (Hooijerink *et al.*, 1991; Kim *et al.*, 1991). The technique in some form has been used for the determination of aromatic sulfonic acid from aquatic environment (Cocheci and Gitye, 1989; Kim *et al.*, 1991). In other cases determination of several industrial chemicals, insecticides, herbicides, phenoxy acids from soil, drinking water and other aquatic environments is reported (Braithwaite and Smith, 1990; Marvin *et al.*, 1991; Kim *et al.*, 1991).

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UV absorption patterns correlate with the structure of the organic molecules. Although aliphatic hydrocarbons also show UV absorption pictures particularly if they have resonance in the molecular structure due to alternate double and single bonds, however aromatic hydrocarbons or partially aromatized molecules give a characteristic UV absorption picture. These spectra can be used as indicators of qualitative and quantitative evaluation of the benzenoid compounds (Kurzer and Chapman, 1989). A large number of insecticides contain benzene or substituted benzene rings as a part of their molecular structure. These pesticides have usually two fates, either these get entry into food chains and thus reach non target animals causing health hazard or they are biodegraded by microorganisms present in soil or water. It is difficult to estimate the amount present in the environment due to high cost instruments involved. UV spectra of the insecticides were obtained to correlate the amount and type of the insecticide present in the media used for the growth of presumptive insecticide degrading bacteria. The study, use and improvement of insecticide degrading bacteria would aid in removing insecticides from the environment.

MATERIALS AND METHODS

UV absorption spectra of phenol and benzoate

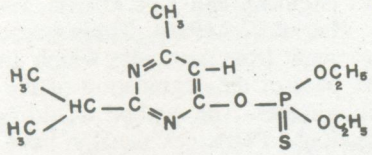
Phenol was taken as an initial material to take UV absorption spectra. A U2000-Hitachi spectrophotometer was used to scan the various wavelengths for determination of maximum UV absorption of phenol. Phenol was dissolved in water to prepare 10 ppm solution. Distilled water was used as a blank for reference. Different concentrations of phenol were used for UV absorption spectra to check the level or wavelength range of maximum absorption. Another referenced aromatic chemical used was sodium benzoate. All the readings were repeated at least three time to take the means.

UV absorption of insecticides

Commercial samples of locally used organophosphate insecticides namely, diazinon, chlorpyrifos, chlorfenvinphos, fenitrothion, methylparathion, profenofos, methamidophos, dichlorvos and monocrotophos, were used to make various dilutions. Wavelength scan for UV absorption at different λ values was done by taking different values of wavelength on x-axis and their respective absorbance on y-axis. The plot showed the wavelength at which the maximum absorbance was obtained.

RESULTS AND DISCUSSION

The wavelength scan showed maximum absorption of phenol at wavelength ranging from 230-235nm. However, the plateau of the curve ranged from 280 to 195nm with a depression ranging from 235 to 245nm. The characteristic pattern was got repeatedly to exclude experimental error. The peak of the maximum absorption curve increased with the increase in concentration of phenol in the solution. Sodium benzoate solution in water showed maximum absorbance at 235nm, however, the plateau of the curve ranged



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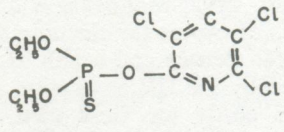
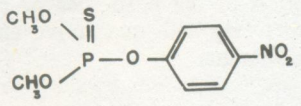
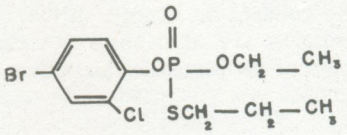


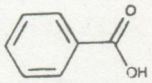
Chlorpyrifos



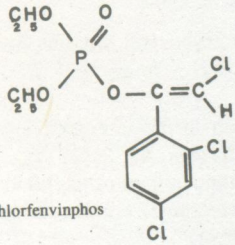
Methyl parathion



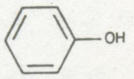
Profenofos



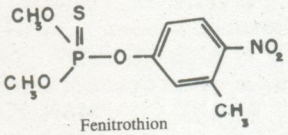
Benzoate



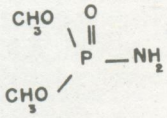
Chlorfenvinphos



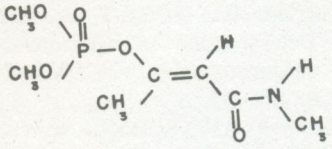
Phenol



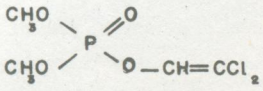
Fenitrothion



Methamidophos



Monocrotophos



Dichlorvos

from 285 to 195nm. There was no depression on the plateau. Diazinon, chlorfenvinphos and fenitrothion showed maximum absorption in a wavelength range of 290-300nm; chlorpyrifos in a range 300-310nm; methylparathion, monocrotophos and profenofos at 300nm; methamidophos and dichlorvos at 290nm.

Aromatic hydrocarbons pollutants get entry into the ecosystems as products of plant metabolism, wastes from industry and agricultural practices. Another big source of pollution and contamination is the pesticide industry. A huge quantity of insecticides is used every year to control pests of agricultural importance but unfortunately these pesticides are not being degraded at the rate to cope with the inflax. Thus many of the pesticides, particularly the insecticides are being accumulated in the ecosystems, food chains and living tissues. Recently attention has been diverted to the microorganisms

important in biodegradation of pesticides (Reincke and Knackmuss, 1988; Eaton and Chapman, 1992; Mahmood *et al.*, 1994; Haq *et al.*, 1996). These microorganisms are the tools of choice for cleaning the environment from pesticides which are becoming an ecological menace and health hazard. The pace of the degradation of the pesticides can be traced by the UV absorption spectrophotometry. The method would be easy, speedy and less costly for these kind of estimations. Pesticides usually have one or more aromatic rings as a part of their molecular structure. These are the basis of UV absorption. Biodegradation, in most of the cases, involve the breakage of benzene rings with the consequent change in the UV absorption of the pesticides or its degradative intermediates. UV absorption patterns would correlate with qualitative and quantitative evaluation of the pesticides and other aromatic pollutants.

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(Received: October 10, 1996)

Punjab Univ. J. Zool., *Vol. 12, pp. 35-42, 1997*

PLASMA FREE AMINO ACID FRACTIONS IN DIFFERENT PHASES OF REPRODUCTION IN DWARF NANNY GOAT*

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Abstract: One dimensional thin layer chromatography was employed to detect free amino acids fractions in various phases of reproduction in dwarf goat. Cystine of band 1, observed in amino acids standard, could not be separated in samples. No appreciable variation was observed in arginine, histidine, lysine, glutamine and asparagine fractions of band 2, tryptophan, valine and methionine of band 7 and phenylalanine and 3, 4-dihydroxyphenylalanine of band 8 throughout the estrous cycle. Glycine and serine of band 3 were significantly reduced, compared to proestrus, in estrus, metestrus and diestrus phases. Threonine of band 4 and leucine and iso-leucine of band 9 were significantly enhanced in estrus compared to proestrus phase. The level was, however, restored in succeeding phases of estrous cycle. Alanine of band 5 and tyrosine of band 6 were markedly reduced in estrus compared to proestrus phase. The level was found rehabilitated in following metestrus and diestrus stages.

Arginine, histidine, lysine, glutamine and asparagine of band 2 were found to be markedly enhanced in early, mid and late gestation compared to prepregnancy estrus. Glycine and serine of band 3 were significantly depressed in mid and late gestation compared to prepregnancy estrus. Threonine of band 4 appreciably declined in early but markedly enhanced in mid and late gestation compared to prepregnancy estrus. Tyrosine of band 6 remained almost unaffected in early and mid, however, reduced in late gestation in comparison with prepregnancy estrus. Tryptophan, valine and methionine of band 7, phenylalanine and 3, 4-dihydroxyphenylalanine of band 8 and leucine and iso-leucine of band 9 remained almost in the range of prepregnancy estrus throughout the entire course of pregnancy.

Arginine, histidine, lysine, glutamine and asparagine of band 2 and threonine of band 4 were appreciably enhanced in mid and late compared to early lactation. Glycine and serine of band 3 reduced in mid and late but elevated in advanced lactational phase compared to early lactation. Alanine of band 5 and tryptophan, valine and methionine of band 7 depressed significantly in mid and late lactational phases in comparison with early lactation. Tyrosine of band 6 reduced markedly in late compared to early lactation. No significant variation was observed in phenylalanine and 3, 4-dihydroxyphenylalanine of band 8 and leucine and iso-leucine of band 9 throughout the entire course of lactation.

Key words: Dwarf goat, amino acid fractions, estrous cycle, pregnancy, lactation.

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*A part of the study conducted under Pakistan Science Foundation Research Project No. P-PU/Agr (138).

INTRODUCTION

Amino acids are the crucial nutrients in pregnancy and lactation. Plasma amino acid pools show important variations throughout the gestational period in the rat and these are net consequences of maternal adaptations to the increased metabolic needs (Pastor-Anglada *et al.*, 1986). In spite of eventual catabolic phase during the last third of pregnancy, nitrogen retention seems to increase in pregnant rats. Furthermore, high placental transfer of amino acids maintains an adequate nutrient supply to the fetuses (Remesar *et al.*, 1987). Alanine and glycine constituted the largest proportions of all the 17 amino acids in the blood plasma in high pregnant sows (Jezkova *et al.*, 1990). In analyses, in healthy mothers after delivery, it was suggested that reduced placental gradient of amino acids could be one of the compensatory mechanisms to facilitate their adequate supply to fetus in human (Boersma *et al.*, 1980). Steingrimsdottir *et al.* (1993), as evaluated in human uterus, consider that amino acids are in excess and are not needed in anabolic processes or as a fuel.

Lactation, specifically, exerts a rapid and formidable demand on amino acids supply and skeletal muscle protein is the major source of endogenous amino acids (Mepham, 1987). Pamblanco *et al.* (1989) observed increase in amino acid fractions in earlier phase and showed decrease in the later phase of lactation.

In a monitoring of free amino acids in plasma and muscle, in dairy cow, it was noticed that compare to precalving concentrations methionine, phenylalanine, glutamine and glycine decreased by 16, 24, 25 and 25%, respectively, and the muscle protein was degraded for supply of amino acids to the udder, particularly, glycine is potentially limiting for milk protein synthesis in high yield cow. Looking into the availability of an amino acid and its consequent appearance in milk, in certain studies, raise the question to the type and source of amino acid precursors by tissue for protein synthesis (Backwell *et al.*, 1996).

Thus there is a need to follow the mechanism of metabolism of various nutrients during the entire period of reproduction. Hence, the present study was planned to observe the pattern of free amino acids fractions in plasma of dwarf goat, as a ruminant model, during various phases of its reproduction.

MATERIALS AND METHODS

Free amino acids in plasma were determined by one dimensional thin layer chromatography (Berry, 1971). Plasma free amino acids were extracted with redistilled ethanol. The extract was dried and amino acids were reconstituted in 50% isopropyl alcohol. The fractions were separated on precoated cellulose plates (5632-D. Fertigplatten cellulose, Merck). Different fractions of separated amino acids were visuallized in the form of blue violet bands while reacting with ninhydrin. Plates were immediately photographed and their images were saved on a floppy disk with image store 5000 Gel Documentation System. Amino acids in each band were identified by their Rf values. The quantification of separated amino acids fractions was carried out by

UVP gel base software programme. The system, while reading the TLC images, provides the data of the total area covered by each of the bands displayed in the form of peaks in the histogram. The data was analyzed statistically using one way analysis of variance ($P<0.05$) and employed in finding the enhancement or reduction and the appearance or disappearance of particular amino acids fractions for comparison among different reproductive phases.

RESULTS

Seventeen amino acids fractionated in eight bands, by thin layer chromatography, were detected in various phases of reproduction in dwarf goat. Cystine of band 1, observed in amino acids standard, could not be separated in samples, whereas, proline and hydroxyproline were not visible by ninhydrin reagent but they appeared as yellow spots on the plates.

Estrous cycle

No appreciable variation was observed in arginine, histidine, lysine, glutamine and asparagine fractions, throughout the different stages of estrous cycle. Glycine and serine, on the other hand, were found to be significantly reduced by 41% in estrus compared to proestrus phase. In metestrus, an elevation of 36% was exhibited by glycine and serine fractions when compared to the estrus phase, however, there was still 20% lowering in comparison with the proestrus phase. No change was exhibited by both of these fractions in diestrus compared to metestrus phase but the fractions were found to be significantly lowered by 28% when compared to the proestrus phase of the estrous cycle. Threonine was found to be significantly and pronouncedly enhanced by 128% in estrus compared to proestrus phase. The level returned in the range of proestrus in the succeeding metestrus phase, however, it was still 52% lower as compared to the estrus phase and remained in the same range even in diestrus phase. A significant decline of 24% as compared to proestrus, was exhibited by this fraction in the succeeding metestrus and diestrus stages.

Tyrosine was found to be appreciably reduced by 34% in estrus compared to proestrus phase. The level returned to the proestrus range in metestrus phase although it was found to be 56% higher as compared to estrus stage. No significant change was observable in the concentration of tyrosine fraction in diestrus phase. Tryptophan, valine and methionine fractions did not exhibit any appreciable variation throughout the entire course of estrous cycle with the exception of a 20% decline observed in metestrus compared to the estrus phase. The level of phenylalanine and 3-4, dihydroxy phenylalanine was found to be the same in all of the four stages of estrous cycle. Leucine and iso-leucine were found to be markedly elevated by 88% in estrus compared to proestrus phase. The level was found to be rehabilitated in metestrus phase, however, it was almost 43% lower as compared to the estrus phase. Pronounced elevations of 54 and 63% were observed in diestrus compared to metestrus and proestrus stages, respectively (Fig.1 A).

Pregnancy

Concentrations of arginine, histidine, lysine, glutamine and asparagine fractions remained unaffected throughout the entire course of pregnancy.

Glycine and serine were found to be 27% lower in mid and 32% lower in late compared to early gestation. The lowerings were, however, found to be 37% and 57% respectively, in mid and late gestation when compared to prepregnancy estrus. A marked reduction of 33% was exhibited by threonine fraction in early pregnancy compared to prepregnancy estrus. Fraction was found to be pronouncedly enhanced by 177% in mid compared to early gestation. A further enhancement of 29%, compared to mid gestation, was observable in late gestation. These enhancements in mid and late gestation were even more intensified when compared to prepregnancy estrus. Alanine remained almost unaffected in early pregnancy compared to prepregnancy estrus, however, a 39% significant reduction, compared to early gestation, was observed in mid gestation. The level remained within the same range even in late gestation.

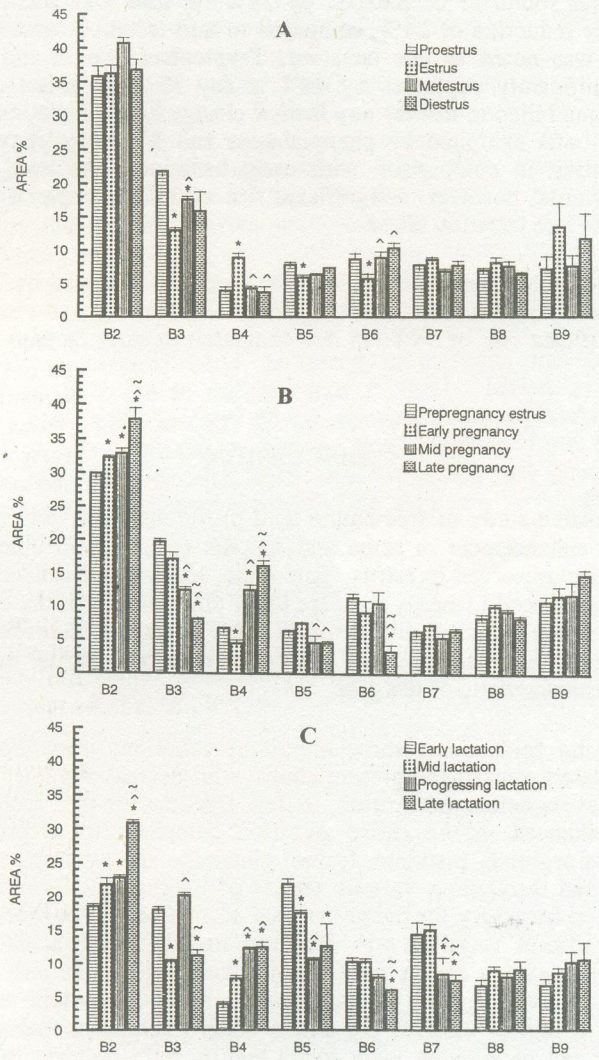
Tyrosine was found to be 20% reduced in early pregnancy compared to prepregnancy estrus. Fraction remained within the same range in mid pregnancy, however, a marked decline of 68%, compared to mid pregnancy, and 70% compared to prepregnancy estrus, was observed in late gestational phase. No significant variation was observed in tryptophan, valine and methionine fractions throughout different phases of pregnancy except in mid gestation where a significant decline of 26%, compared to early gestation, was noteworthy. Phenylalanine and 3-4, dihydroxy phenylalanine were found elevated by 20% in early gestation compared to prepregnancy estrus. The level remained almost unaffected in mid gestation and even restored to prepregnancy range in following late gestation.

Leucine and iso-leucine remained almost in the same range in prepregnancy estrus, early pregnancy and mid pregnancy, however, a 25% elevation, compared to mid pregnancy, was observed in late gestational phase (Fig.1 B).

Lactation

Arginine, histidine, lysine, glutamine and asparagine fractions remained almost unaffected throughout the entire course of lactation with the exception of 35% elevation observed in late lactation as compared to day 35 of mid lactation.

Glycine and serine were found to be highly suppressed by 42% at day 28 of mid lactation. Conversely, with advancing lactation (day 35), a highly significant elevation of 94%, compared to day 28 of lactation, was noteworthy which was found to be reduced by 45% in late lactation. Threonine was found to be highly significantly enhanced by 98% in mid (day 28) compared to early lactation. Further elevation of 58% was observed at day 35 of lactation. The level was 114% higher when compared to early lactation and remained within the same range in succeeding late lactational phase. Significant reductions of 20% and 52%, in alanine fraction, were observed at day 28 and 35 of lactation, respectively, as compared to early lactation. The fraction did not exhibit any further alteration in its concentration in late lactation and remained within the range



B2 arginine, histidine, lysine, glutamine and asparagine; B3 glycine and serine; B4 threonine; B5 alanine; B6 tyrosine; B7 tryptophan, valine and methionine; B8 phenylalanine, 3-4, dihydroxy phenylalanine; B9 leucine, iso-leucine.

Average percent areas covered by various amino acids fractions resolved into eight bands (B2-B9), in different stages of estrous cycle (A), pregnancy (B) and lactation (C) in goat. Values are mean ± SEM. *, ˆ and ˜ Significances ($P < 0.05$) in relation to proestrus, estrus & metestrus (A); pre, early & mid pregnancy (B); early, mid & progressing lactation (C), respectively.

of mid lactational (day 35) value.

Tyrosine was found to be reduced by 23%, in mid (day 35) compared to early lactation. Further reduction of 24%, compared to mid lactation, and 41%, compared to early lactation, was noted in late lactation. Tryptophan, valine and methionine were found to be significantly depressed by 44% at day 35 of lactation compared to early lactation. Fractions failed to display any further change in late lactation phase. A marked increase of 34% was exhibited by phenylalanine and 3-4, dihydroxy phenylalanine at day 28 of lactation in comparison with early lactation. The level remained almost unaffected afterwards, however, a significant rise of 37%, compared to early lactation, was observable in late lactation phase.

Leucine and isoleucine were found to be markedly elevated by 28% at day 28 of lactation compared to early lactation. No further alteration was observed afterwards, however, a significant rise of 58%, in late compared to early lactation, was noteworthy (Fig.1 C).

DISCUSSION

In the extensive study of free amino acid profle and their patterns, in the cycling goat, a specific enhancement in some and specific reduction in other free amino acid fractions have been observed in estrus. Threonine, leucine and isoleucine were found to be significantly enhanced in estrus compare to all other phases of the cycle. On the other hand, glycine, serine, alanine and tyrosine were markedly depressed. The pertinent variations in the different fractions of amino acids, during estrus, may be linked with multiovulatory characteristic of the goat.

Plasma amino acids pools show important variations throughout the gestational period, in the Dwarf goat of the present study, with decreased values at mid pregnancy and recovered levels before parturition. A few fractions of free amino acids remained exceptionally enhanced in the entire gestation compared to prepregnancy estrus as concentrations of arginine, histidine, lysine, glutamine and asparagine were found to be markedly enhanced throughout various phases of pregnancy. Their increased presence may be considered necessary for the gestational adaptation by the breed to carry multiple fetuses. No significant variation was observed in free amino acids fractions in early compared to prepregnancy except for the reduction in threonine and tyrosine fractions. This indicated the involvement of these amino acids in protein anabolism of early pregnancy. The levels of most of the amino acids fractions including glycine, serine, tyrosine, valine and methionine were found significantly reduced in mid compared to early gestation. This decrease in amino acids levels, at mid pregnancy, is mainly due to the changes in gluconeogenic amino acid group. The decreased trend of maternal circulatory amino acids may also be attributed to their rapid clearance after being uptaken by the fetus. It appeared as if the availability of these amino acids through catabolism in the cireulation could not overtake the fraction's clearance by the fetus. In late gestation, glycine, serine and tyrosine fractions were markedly reduced while threonine, leucine and isoleucine were found to be appreciably enhanced. The trend of these fractions, in late pregnancy, may be attributed to the catabolic role of these in late

pregnancy. The discriminaing behaviour of free amino acid fractions has been reported by other studies. In pregnant rabbits, when hyperaminoacidemia occurs, a greater amount of gluconeogenic amino acids (glycine and serine) would escape the liver, suggesting a higher availability of these for the fetus (Pere *et al.*, 1994).

In the present study, in dwarf goat, significantly elevated concentrations of glycine, serine, alanine, tyrosine, tryptophan, valine and methionine were encountered in the entire period of lactation when compared to preparturition levels. Increased levels of these amino acids in plasma are indicator of essential amino acids availability for the lactating tissue in cow (Shmanenkov, 1986). Lopez-Tejero *et al.* (1989) also reported increased levels of plasma essential free amino acids at the peak of lactation. Moreover, the levels of arginine, histidine, lysine, glutamine, asparagine, threonine, phenylalanine, leucine and isoleucine were found to be markedly reduced in early lactation signifying their extensive uptake from plasma for the synthesis of protein rich clostrum. Several amino acids fractions including glycine, serine, alanine, tyrosine, tryptophan, valine and methionine were found significantly depressed in mid and late compared to early lactation indicating their use in milk protein synthesis. Meijer *et al.* (1985) while monitoring free amino acids, in dairy cows, reported that the plasma concentrations of methionine, phenylalanine, glutamine and glycine decreased from 6 to 15 weeks of lactation. The decrease corresponded to the order in which they generally appear to be limiting for milk protein synthesis. Probably the availability of these fractions is not sufficient for satisfactory let down of milk.

Lactation phase thus exerts heavy demand on maternal plasma metabolites for the production of milk and such situation is of greater magnitude in ruminants such as cow, sheep and goat (Baldwin and Plucinski, 1977). Lactation, specifically, exerts a rapid and formidable demand on amino acids supply and skeletal muscle protein is the major source of endogenous amino acids (Mepham, 1987). Differences in the contents and changes in free amino acids levels, during lactation, among the groups were observed by Pamblanco *et al.* (1989). Amino acids fractions increased in the earlier phase and showed decrease in the later phase of lactation. Increased levels of total plasma free amino acids are present during lactation of dwarf goat (Bogin and Lappis, 1980).

On the overall, in the present investigation, it is realized that the status of free amino acids fractions is not favourable for adequate let down of milk in this breed of goat and the metabolites setup of the goat adapted during pregnancy fails to shift to an efficient lactational level. The restoration of the levels of certain free amino acids fractions, peculiar to pregnancy, including arginine, histidine, lysine, glutamine, asparagine, threonine, phenylalanine, leucine and isoleucine, in mid lactation, soon after a short early lactation, supports this apprehension. This probably renders the lactation phase poor in performance.

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(Received: June 25, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 43-48, 1997

PLASMID-MEDIATED ANTIBIOTIC RESISTANCE IN CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA*

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Abstract: A total of eighteen *Pseudomonas aeruginosa* isolated from CMH Muzaffarabad, were screened for their resistance to the antibiotics, Ampicillin, Chloramphenicol, Doxycycline, Erythromycin, Gentamicin, Kanamycin, Minocycline, Nalidixic acid, Penicillin, Streptomycin, Urixin and Velosef. Generally, the isolates showed the highest frequency of resistance to Penicillin and lowest to Velosef, followed by Doxycycline and Erythromycin. The resistant isolates showed eight patterns of antibiotics resistance at a level as high as 1000 μg/ml. Some of the antibiotic determinants were cured by acridine orange treatment, indicating the widespread antibiotic resistance is mediated through plasmids among *Pseudomonas aeruginosa*.

Key words: Antibiotic resistance, plasmid, bacterial isolates.

INTRODUCTION

Pseudomonas aeruginosa is notorious for its resistance to most commonly used antimicrobial agents. It appears to be a common hospital pathogen because of its resistance to a number of antibiotics, including, Carbenicillin, Gentamicin, Tobramycin (Tsakris *et al.*, 1992). It appears that widespread use of such drugs has resulted in the development of a variety of R-factors which can inactivate these antibiotics by phosphorylation, adenylation, acetylation or by a combination of them (Erova *et al.*, 1989; Eleanor and Sallch, 1992). Montelli *et al.* (1989) showed the highest level of resistance against 23 antimicrobial agents in *Pseudomonas* strains. Moreover, the authors also found a significant increase in resistance to some aminoglycosides and beta-lactam compounds. *P. aeruginosa* have also shown resistance to extended spectrum cephalosporins (Patrice *et al.*, 1993).

Evidences have been provided from several countries about the misuse and abuse of antibiotics and this indiscriminate use of these drugs is contributing to a world-wide increase in resistant strains of bacteria (Schneider and Schweisfurth, 1991). Widespread use of these antibiotics has failed to eradicate infections and instead an increase in antibiotic resistant bacteria have been observed all over the world (Maple *et al.*, 1989; Nazarov *et al.*, 1989; Dakshinkar *et al.*, 1992; Fung-Tome *et al.*, 1993).

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The present study deals with high levels of multiple antibiotic resistance of *P. aeruginosa* and their plasmid associated nature (Tsakris *et al.*, 1992; Blahova *et al.*, 1992; Padilla and Vasquez, 1993). R-plasmids like other extrachromosomal elements, can be lost spontaneously from the host cell because of some errors in replication or segregation (Watanabe and Lyang, 1962b). These losses (elimination or curing) can be increased by treating the host cell with acridine orange (Watanabe and Lyang, 1962a). Thus plasmid loss or curing can be used as a criterion for determining plasmid associated nature of antibiotic resistance.

In Pakistan, like other developing countries there is a general increase in antibiotic resistance especially to all commonly used antibiotics (Khursheed and Khatoon, 1984; Naqvi *et al.*, 1990; Ansari and Khatoon, 1994). Hence there is a need to monitor the prevailing levels of resistance so that an effective system for bacterial therapy can be developed.

MATERIALS AND METHODS

Pseudomonas aeruginosa strains were isolated from patients at Combined Military Hospital (CMH) Muzaffarabad. Bacterial cultures were maintained in freezing glycerol nutrient media at -20°C. For routine experiments, the cultures were maintained on nutrient agar plates at 4°C and subcultured bimonthly. Difco (USA) nutrient broth and agar were used for the screening of cultures for antibiotic resistance.

Antibiotics

Twelve antibiotics were used in these studies. These were, Ampicillin (A), Chloramphenicol (C), Doxycycline (D), Erythromycin (E), Gentamicin (G), Kanamycin (K), Minocycline (M), Nalidixic acid (N), Penicillin (P), Streptomycin (S), Urixin (U) and Velosef (V). A stock solution (10 mg/ml) of each antibiotic was made in distilled water. Chloramphenicol was dissolved in ethanol. These stock solutions were used for calculating the dose of each antibiotic used in resistance experiments. All solutions were sterilized by millipore (0.45 mμ) filters and refrigerated.

Antibiotics susceptibility testing

The cultures obtained were further identified on the basis of pigment production. The pure isolates were subjected to antibiotic resistance screening by replica plate method (Lederberg and Lederberg, 1952). For this purpose, a broth culture of the test strain was plated on nutrient agar plate to obtain isolated colonies. A few colonies were picked on to a master plate, incubated overnight and replicated on nutrient agar plates containing different concentrations of all the antibiotics. The highest concentration of an antibiotic showing growth of all the replicated clones was taken as the resistant level of the strain for the particular antibiotic.

Acridine orange mediated plasmid curing

The method of Hirota (1960) was followed for this purpose. A small inoculum ($2x10^{-2}$ - $5x10^{-2}$ bacteria) was added to varying concentrations of acridine orange broth and incubated at 37°C overnight. Cultures containing the highest concentration of acridine orange in which growth was clearly visible were diluted and spread on nutrient agar plates containing appropriate antibiotic.

RESULTS

The antibiotic resistance was screened against eighteen clinical isolates of *P. aeruginosa*. Overall 86% of strains were resistant to Penicillin (P), 80% to Kanamycin (K), 76% to Gentamicin (G), 75% to Ampicillin (A), 73% to Streptomycin (S) and Urixin (U), 67% to Chloramphenicol (C) and Doxycycline (D), 66% to Minocycline (M), 65% to Velosef (V), 63% to Nalidixic acid (N) and 60% to Erythromycin (E).

The results of MICs against twelve antibiotics resistance were obtained and indicated in a comparative account of antibiotics resistance of isolates at differnt levels in Table 1. Generally, the isolates showed the highest frequency of resistance to Penicillin (55%) and the lowest to Velosef (5%) followed by Doxycycline (11%) at 1000 μg/ml (Table I). There was a slight decrease in the number of Penicillin resistant isolates at the levels of 250-1000 μg/ml compared with the level of 25 μg/ml. At 500 μg/ml level the isolates showed a considerable decrease in the resistance frequency of almost all the antibiotics tested.

Table 1: Occurrence of antibiotic resistance of *P. aeruginosa* at six different concentrations.

Antibiotics	Number of resistant cultures at concentrations of (μg/ml)					
	25	50	100	250	500	1000
Ampicillin	17	17	17	16	8	6
Chloramphenicol	17	16	15	14	6	5
Doxycycline	18	17	16	13	7	2
Erythromycin	16	14	13	11	8	3
Gentamicin	18	18	17	14	10	5
Kanamycin	18	18	18	17	9	7
Minocycline	17	16	15	12	7	4
Nalidixic acid	16	15	14	13	6	4
Penicillin	18	18	18	17	12	10
Streptomycin	18	18	17	13	9	5
Urixin	17	17	16	13	9	7
Velosef	16	16	15	14	8	1

Multiple antibiotic resistance among *P. aeruginosa* isolated from clinical sources is not uncommon. The strains screened were 88% resistant to three or more of the antibiotics at 100 μg/ml, 78% were resistant to three or more antibiotics at 250 μg/ml, 44% were resistant to three or more antibiotics at 500 μg/ml and 28% were resistant to one or more antibiotics at 1000 μg/ml. The resistant cultures showed eight differnt patterns of antibiotics resistance at a level as high as 1000 μg/ml (Table II). Among these, AKPU resistance was found to be the most common.

Table II: Antibiotic resistance pattern at 1000 μg/ml in *P. aeruginosa* isolated from different clinical specimens.

Pattern Number	Resistance pattern	No. of cultures
1	ACDEGKMNPSV	1
2	CEGKMNPSU	1
3	CEGKMPSU	1
4	ACGMPSU	1
5	ACDGNP	1
6	AKPU	3
7	CNPU	1
8	KPU	1

Abbreviations used: Ampicillin, A; Chloramphenicol, C; Doxycycline, D; Erythromycin, E; Gentamycin, G; Kanamycin, K; Minocycline, M; Nalidixic acid, N; Penicillin, P; Streptomycin, S; Urixin, U; Velosef, V.

Loss of antibiotic resistance after plasmid curing

Two representative multiple antibiotic resistant *P. aeruginosa* cultures (patterns 1 and 2) were selected for plasmid curing. Out of 100 colonies each from two treated cultures some had lost the resistance to one or the other antibiotics. However, a total loss to Velosef was found in one of the cultures.

DISCUSSION

The results indicate that antibiotic resistance among indigenous clinical samples of *P. aeruginosa* is very common. Of the strains screened for resistance, 44% were resistant to three or more antibiotics at 500 μg/ml and 28% were resistant to three or more antibiotics at 1000 μg/ml. These findings are similar to those reported by earlier workers (Eleanar and Sallch, 1992; Tsakris *et al.*, 1992; Fung-Tome *et al.*, 1993; Patrice *et al.*, 1993). The resistance to doses as high as 1000 μg/ml is alarming because if bacteria become resistant to such high levels of antibiotic, disease treatment with antibiotics would become quite difficult and may not be possible.

A total resistance towards Penicillin was exhibited by *P. aeruginosa* (Dakshinkar *et*

al., 1992). In this study the highest frequency of resistance was against Velosef at 1000 μg/ml and it seems to be the most effective antibiotic for treating infections of *P. aeruginosa* with high doses. The other effective antibiotics at 1000 μg/ml were Doxycycline and Erythromycin.

In the present study the maximum number of the resistant cultures were found at the level of 25 μg/ml (95%). Only 4.6% of the cultures were sensitive to all the twelve antibiotic tested. We also observed very high resistance levels in 18 strains for which MICs of the antibiotics were tested. The multiple antibiotic resistance may result from the selection of resistant mutants through the widespread use of antibiotics.

In view of the overall high incidence of multiple antibiotic resistance among *P. aeruginosa*, the possibility of the presence of R-plasmids was explored. The loss of resistance to single or multiple antibiotics after acridine orange treatment of cultures points to the fact that observed antibiotic resistance was plasmid borne. Neither of the two cultures showed a loss of resistance to all the antibiotics simultaneously. This shows that different plasmids determine the resistance against different antibiotics. Plasmid analysis undertaken by Tsakris *et al.* (1992) showed that *P. aeruginosa* harbour plasmids ranging in size from 20 to 100 Mda and the larger plasmids (100 Mda) encoded high level resistance to Gentamiccin and Tobramycin, as was also seen to Kanamycin and Streptomycin by Blahova *et al.* (1992) and to Ampicillin and Chloramphenicol by Padilla and Vasquez (1993).

Loss of Velosef resistance in all the colonies of two of the cured cultures could be due to the fact that the corresponding plasmid may be very small and fragile which could not resist the curing treatment in all the cells. Low percentage of curing in other resistant determinants was observed in these studies. Analogous results have been reported in *Aerobacter aerogenes* by Khatoon and Mohammad (1986) and in *Staphylococci* by Rasool *et al.* (1987). Since curing was not observed in all the resistant determinants it may be presumed that some plasmids were present in integrated state and hence stable as has been suggested by Hirota (1960).

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(Received: May 22, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 49-58, 1997

STUDIES ON THE DIGESTIVE TUBE OF THE GENUS *AMITERMES* SILVESTRI WITH A NOTE ON ITS DISTRIBUTION

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Abstract: Configuration of digestive tube and enteric valve armature of termite species, *Amitermes belli* (Desneux), *A. dentatus* (Haviland) and *A. paradentatus* Ahmad are described. In genus *Amitermes*, the enteric valve seating is mid-dorsal. Enteric valve cushions are tube like with nipple like scales. Scales at anterior end are with longer spines than at posterior end, and tips of the scales are provided with pimple like projection. *Amitermes belli* (Desneux) and *A. paradentatus* Ahmad have almost the same configuration of the digestive tube; but in *A. dentatus* (Haviland) mixed segment is present. Zoogeographical distribution of 81 species of genus *Amitermes* is listed. The species of genus *Amitermes* are distributed as follow: Australian region (27), Ethiopian region (30), Indomalayan (6), Nearctic (8), Neotropical (7), Palaearctic (3). It is postulated that the genus *Amitermes* orignated in the Ethiopian region.

Key words: *Amitermes*, enteric valves, zoogeographical distribution.

INTRODUCTION

In Pakistan, genus *Amitermes* is represented by three species; *A. baluchistanicus* Akhtar, *A. belli* (Desneux) and *A. paradentatus* Ahmad. Akhtar (1972) described *A. baluchistanicus* from Baluchistan, since then it has not been reported from any other part of Pakistan. Similarly, *A. paradentatus* was recorded only once from Karachi. On the other hand, *A. belli* (Desneux) is widely distributed in Pakistan and shows great adaptability to different ecological conditions. According to Emerson (1955), genus *Amitermes* is cosmopolitan, and the centre of origin of genus is obscure, because most of the species are found in Africa, but the most primitive are Indomalayan.

In the present paper digestive tube features have been studied in detail and the study is based upon following species: *A. belli* (Desneux), *A. dentatus* (Haviland) and *A. paradentatus* Ahmad. Besides, zoogeographical is discussed.

MATERIALS AND METHODS

The study is based on the material collected by Dr. Muzaffar Ahmad and Dr.

0079-8045/97/0001-0047 $ 03.00/0

Muhammad Saeed Akhtar, University of the Punjab, Lahore. The specimens are presently in the custody of first author.

The position of alimentary canal of the worker was observed through the body wall of an unopened specimen. Later on, specimen was dissected under Leitz stereoscopic microscope with built-in magnification changer, and the gut was exposed. After noting the coiling of the gut and malpighian tubules attachment, the gut was uncoiled and straightened for making diagram with the help of Camera Lucida. To study the armature of enteric valve cushions, slides of the cuticle were prepared.

RESULTS

Systematic accounts

Genus ***Amitermes***

Amitermes belli *(Desneux)*

Configuration of digestive tube

Dorsal view (Fig.1a)

The following structures are visible in the digestive tube (*in situ*), oesophagus (O); crop (cp); gizzard (G); major part of mesenteron (M) forming anticlockwise loop; dilated portion of P_1 towards the left side of abdomen; U-shaped narrow P_2 located towards the right dorsal side of abdomen visible; some part of P_3 below P_2 visible; colon starting within mesenteron loop visible; half part of colon visible on right side; passage of colon into rectum (R) visible in dorsal view.

Ventral view (Fig.1b)

The following structure are visible (*in situ*) in ventral view: oesophagus; crop; posterior part of mesenteron; dilated portion of P_1 is visible on right side of abdomen, P_3 located on left side clearly visible, rectum passing below paunch visible.

Configuration (Fig. 1a-c)

Oesophagus (O) leads into a distinct crop (cp); Armed chitinized gizzard (G); Crop asymmetrical main bulge on the right side of abdomen; Gizzard enters into mesenteron by narrow tube; Mesenteron (M) loops anticlockwise from left to right side of abdomen; P_1 dilated before the narrower P_2 portion; Enteric valve seating mid-dorsal, U-shaped; P_3 dilated anteriorly and tappering posteriorly; Colon lying above the paunch runs backward into rectum.

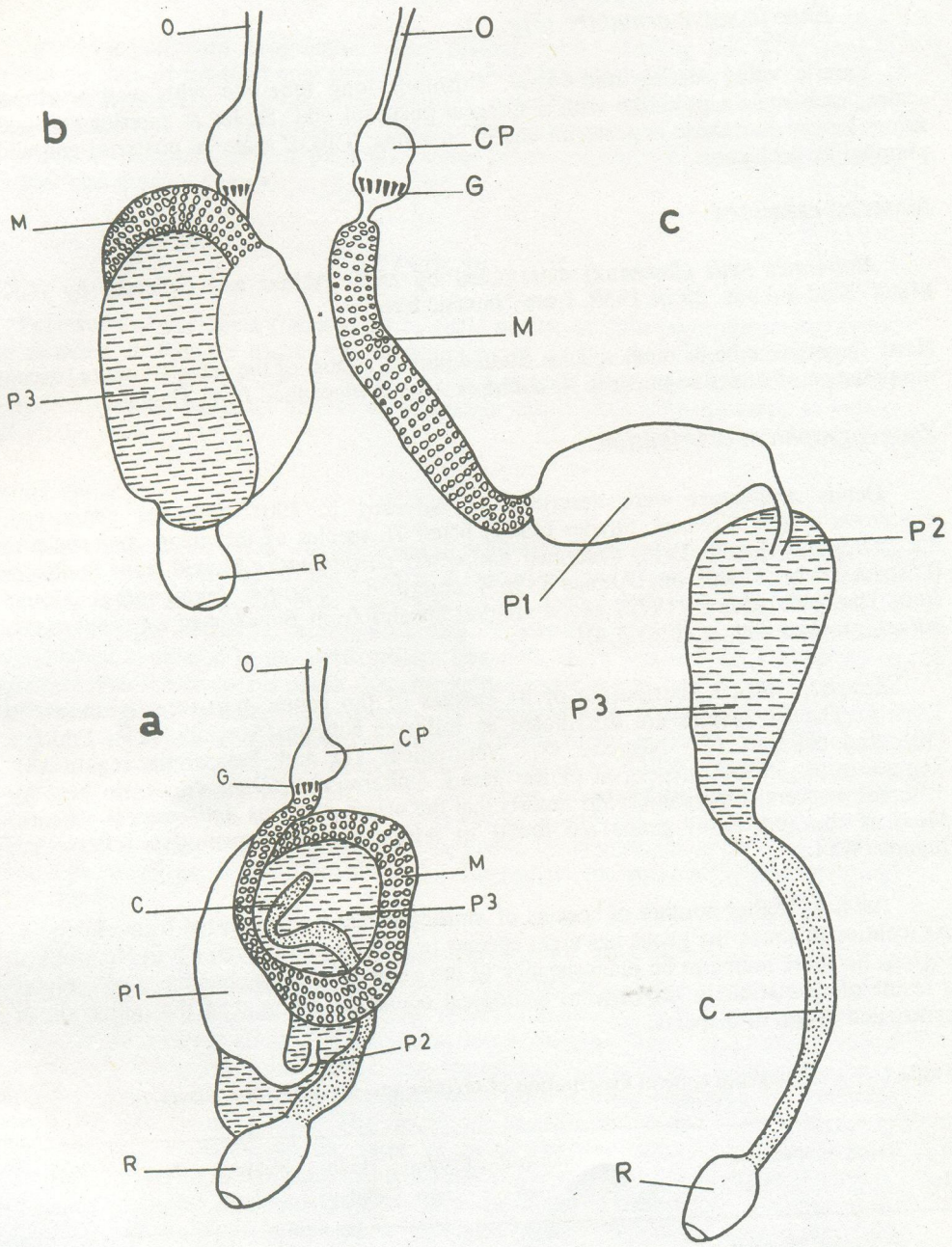

Fig.1(a): Configuration of the digestive tube (*in situ*) of *Amitermes belli*. Dorsal view (X294): O. Oesophagus; Cp. Crop; G. Gizzard; P^1. First proctodeal segment; P^2. Second proctodeal segment; P^3. Third proctodeal segment; C. Colon; R. Rectum.

(b) Ventral view (X294).

(c) Structure of digestive tube of *Amitermes belli* uncoiled (X294).

Enteric valve armature (Fig. 2)

Enteric valve seating mid-dorsal; Cushions long tube-like with well-developed scales, each scale nipple-like with a spine at posterior end. Scales at anterior end with spines longer than those at posterior end of cushions. Tips of scales at posterior end with pimple like projection.

Material examined

Amitermes belli (Desneux) determined by M.S. Akhtar and collected by N.K. Malik, Sind Sukkur, 20.iii.1969, from "Jamen" tree.

Note: Digestive tube of other species studied here is almost of the same structure, except the presence of mixed segment in *A. dentatus* Ahmad (described from Thailand).

Zoogeographical distribution

Genus *Amitermes* were described by Silvestri in 1901 with the generitype, *Amitermes amifer* Silvestri. Snyder (1949) listed 76 species of the genus *Amitermes* in his catalogue. Ahmad (1955) described *Amitermes paradentatus* Ahmad from Budhapur (District Dadu). Later on, Ahmad (1965) described *Amitermes longignathus* Ahmad from Thailand. Akhtar (1972) added another species from Baluchistan *i.e.*, *Amitermes baluchistanicus* Akhtar (Figs.3,4).

Zoogeographical distribution of 81 species of the genus *Amitermes* is shown in Table 1. The 81 species are distributed as follows: Notogea/Australia (27), Ethiopia (30), Indomalayan (6), Nearctic (8), Neotropical (7) and Palaearctic region (3). Regarding origin and dispersion of the genus *Amitermes* different views are held by different workers. Emerson (1955) reports that the origin of genus *Amitermes* is obscure. Most of the species of genus are found in Africa but more primitive relative are Indomalayan.

The next higher number of species of *Amitermes* has been reported from Australia. As mentioned above the genus has eight species in Nearctic and seven (7) in Neotropical region. So there seems to be inconsistancy in the order of species abundance possibly as a result of speciation in response to ecological condition rather than the indication of centre and origin of dispersal.

Table 1: Zoogeographical distribution of termite species of Genus: *Amitermes*.

Sr. No.	Name of species	Australian	Ethiopian	Indomalayan	Nearctic	Neotropical	Palaearctic
1	*A. amifer* Silvestri	-	-	-	-	+	-
2	*A. atlanticus* Fuller	-	+	-	-	-	-
3	*A. baluchistanicus* Akhtar	-	-	+			

4	*A. beaumonti* Banks	-	-	-	-	+	-
5	*A. bechuana* Fuller	-	+	-	-	-	-
6	*A. beli* (Desneux)	-	-	+	-	-	-
7	*A. braunsi* Fuller	-	+	-	-	-	-
8	*A. capicola* Silvestri	-	+	-	-	-	-
9	*A. capite* Hill	+	-	-	-	-	-
10	*A. coachellae* Light	-	-	-	+	-	-
11	*A. colonus* Hill	+	-	-	-	-	-
12	*A. cryptodon* Light	-	-	-	-	+	-
13	*A. darwini* Hill	+	-	-	-	-	-
14	*A. dentatus* (Haviland)	-	-	+	-	-	-
15	*A. dentosus* Hill	+	-	-	-	-	-
16	*A. desertoerum* (Desneux)	-	-	-	-	-	+
17	*A. elongatus* Silvestri	-	+	-	-	-	-
18	*A. emersoni* Light	-	-	-	+	-	-
19	*A. ensifer* Light	-	-	-	-	+	-
20	*A. eucalypti* Hill	+	-	-	-	-	-
21	*A. evuncifer* Silvestri	-	+	-	-	-	-
22	*A. evuncifer-varheterocera* Silvestri	-	+	-	-	-	-
23	*A. excellens*	-	-	-	-	+	-
24	*A. exillis* Hill	+	-	-	-	-	-
25	*A. foreli* Wasmann	-	-	-	-	+	-
26	*A. gallagheri* Chhotani	-	+	-	-	-	-
27	*A. germanus* Hill	+	-	-	-	-	-
28	*A. gunni* Fuller	-	+	-	-	-	-
29	*A. hartmeyeri* Silvestri	+	-	-	-	-	-
30	*A. hastatus* (Haviland)	-	+	-	-	-	-
31	*A. herbertensis* Mjoberg	+	-	-	-	-	-
32	*A. heterognathus* Silvestri	+	-	-	-	-	-
33	*A. kellyi* Fuller	-	+	-	-	-	-
34	*A. kenhardi* Fuller	-	+	-	-	-	-
35	*A. lacertosus* Ghidini	-	+	-	-	-	-
36	*A. latidens* Mjoberg	+	-	-	-	-	-
37	*A. lativentris* Mjoberg	+	-	-	-	-	-
38	*A. laurensis* Mjoberg	+	-	-	-	-	-
39	*A. libertatis* Fuller	-	+	-	-	-	-
40	*A. lompopensis* Fuller	-	+	-	-	-	-
41	*A. londonensis* Fuller	-	+	-	-	-	-
42	*A. longignathus* Ahmad	-	-	+	-	-	-
43	*A. lonnbergianus* (Sjostedt)	-	+	-	-	-	-
44	*A. macrocephalus* Ghidini	-	+	-	-	-	-
45	*A. meridionalis* (Froggatt)	+	-	-	-	-	-
46	*A. meruensis* (Sjostedt)	-	+	-	-	-	-

47	*A. messinae* Fuller	-	+	-	-	-	-
48	*A. minimus* Light	-	-	-	+	-	-
49	*A. minor* Holmgren	-	-	+	-	-	-
50	*A. modicus* Hill	+	-	-	-	-	-
51	*A. murraysburgi* Fuller	-	+	-	-	-	-
52	*A. neogermanus* Hill	+	-	-	-	-	-
53	*A. obeuntis* Silvestri	+	-	-	-	-	-
54	*A. obtusidens* Mjoberg	+	-	-	-	-	-
55	*A. pallidus* Light	-	-	-	+	-	-
56	*A. paradentatus* Ahmad	-	-	+	-	-	-
57	*A. parvidus* Hill	+	-	-	-	-	-
58	*A. parvulus* Light	-	-	-	+	-	-
59	*A. parvus* Hill	+	-	-	-	-	-
60	*A. paucinervius* (Silvestri)	-	+	-	-	-	-
61	*A. perarmatus* (Silvestri)	+	-	-	-	-	-
62	*A. perelegans* Hill	+	-	-	-	-	-
63	*A. ravus* Hill	+	-	-	-	-	-
64	*A. runconifer* Silvestri	-	+	-	-	-	-
65	*A. santschi* Silvestri	-	-	-	-	-	+
66	*A. schoombiensis* Fuller	-	+	-	-	-	-
67	*A. sciangallorum* Ghidni	-	+	-	-	-	-
68	*A. scopulus* Mjoberg	+	-	-	-	-	-
69	*A. seminotus* (Silvestri)	-	+	-	-	-	-
70	*A. silvestrianus* Light	-	-	-	+	-	-
71	*A. snyderi* Light	-	-	-	+	-	-
72	*A. somaliensis* Sjostedt	-	+	-	-	-	-
73	*A. spinifer* Silvestri	-	+	-	-	-	-
74	*A. stephensoni* Harris	-	+	-	-	-	-
75	*A. unidentatus* (Wasmann)	-	+	-	-	-	-
76	*A. vilis* (Hagen)	-	-	-	-	-	+
77	*A. vitiosus* Hill	+	-	-	-	-	-
78	*A. westraliensis* Hill	+	-	-	-	-	-
79	*A. wheeleri* (Desneux)	-	-	-	+	+	-
80	*A. xylophagus* Hill	+	-	-	-	-	-
81	*A. zuurbergi* Hill	-	+	-	-	-	-
Total species in each region		27	30	6	8	7	3

As maximum number of species have been reported from the Ethiopian region, it should be considered its place of origin.

Fig.2 a) Enteric valve armature of cushions of *Amitermes belli* under low magnification (X200); b) Enteric valve armature of cushions of *Amitermes belli* under high magnification (X1000); c) Enteric valve armature of cushions of *Amitermes dentatus* under low magnification (X200); d) Enteric valve armature of cushions of *Amitermes parvidentatus* under high magnification (X1000).

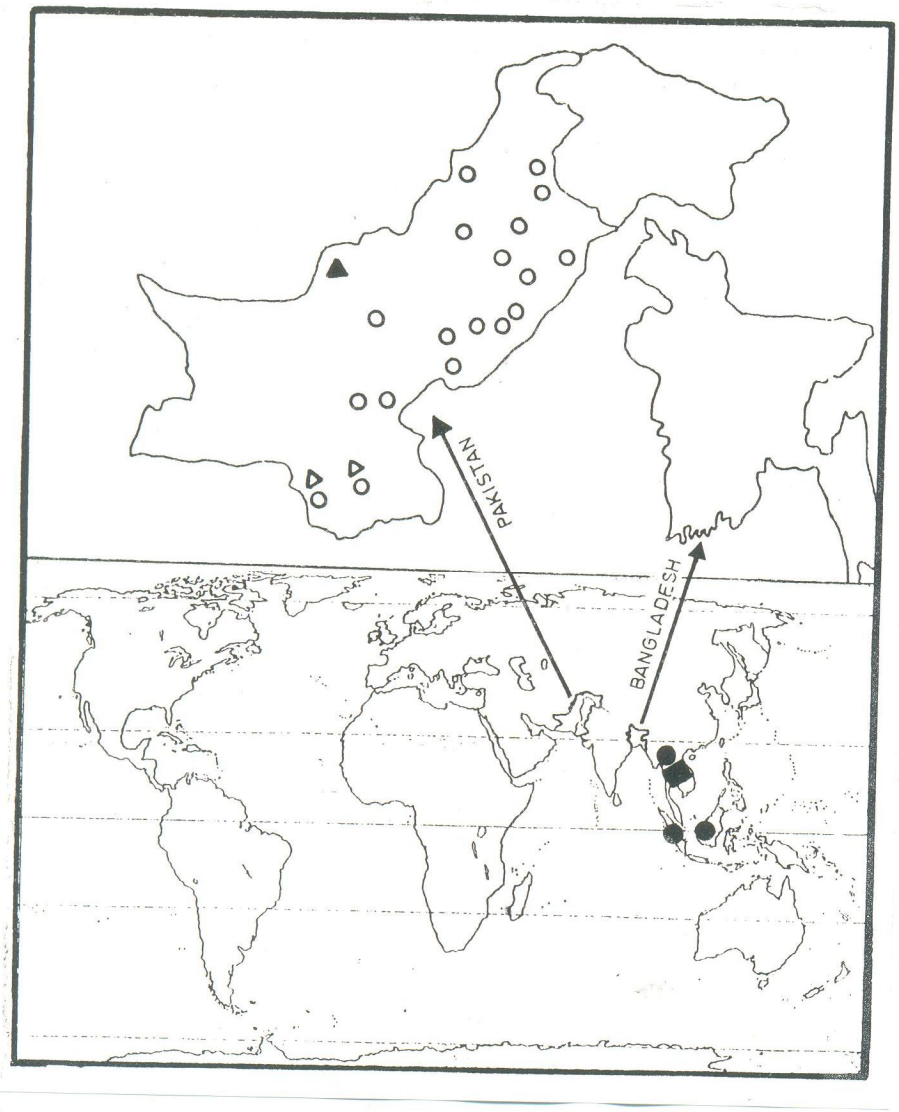

Fig. 3: Distribution of the genus *Amitermes* in the Oriental region.
▲, Amitermes baluchistanicus Akhtar; ◖ , Amitermes belli (Desneux); ● , Amitermes dentatus (Haviland); ■ , Amitermes longignathus Ahmad; △ , Amitermes paradentatus Ahmad.

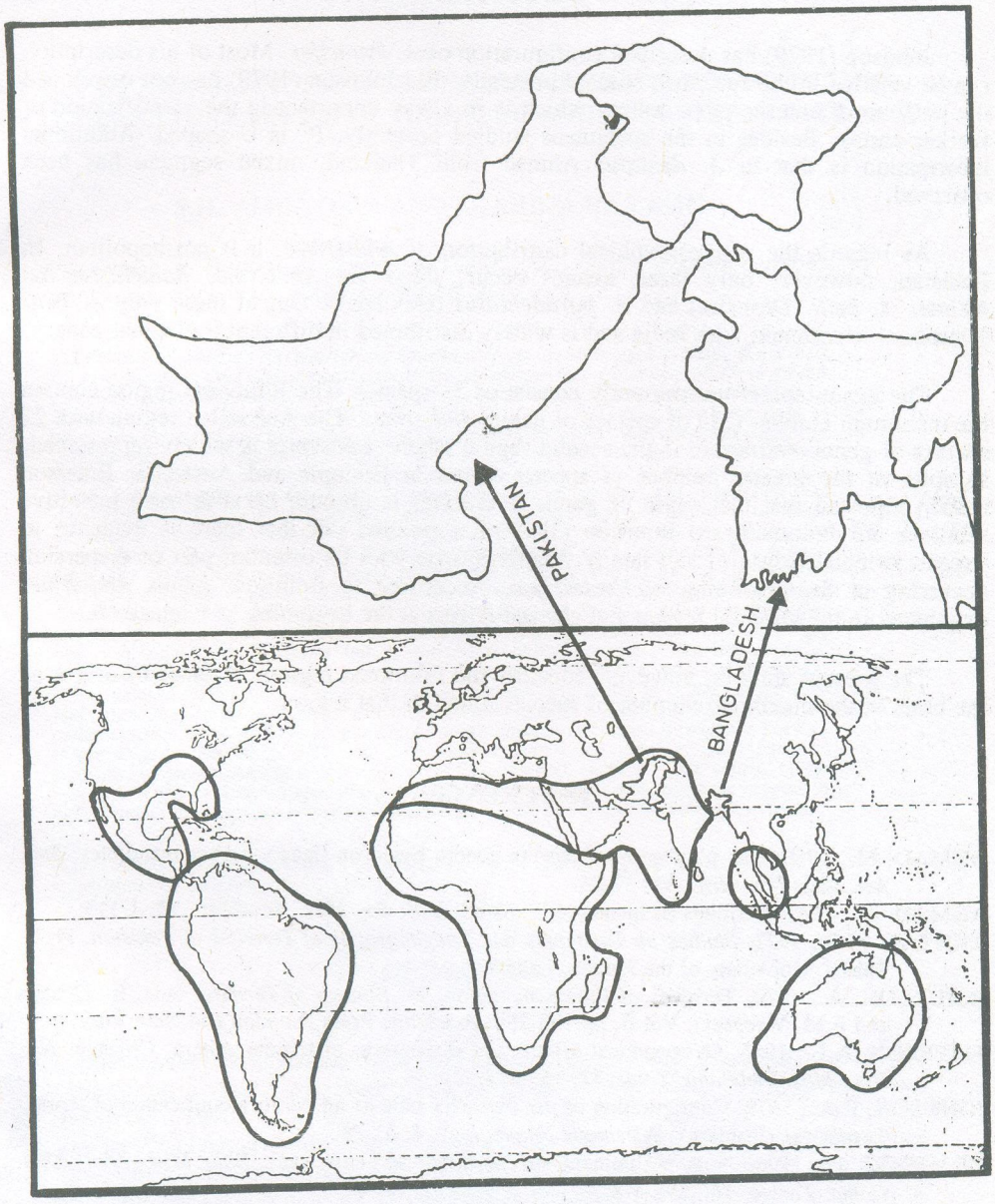

Fig. 4: Distribution of the genus *Amitermes* in different zoogeographical regions.

DISCUSSION

Johnson (1979) has described configuration of *A. evuncifer*. Most of his description can be verified in the specimen studied presently. But Johnson (1979) has not mentioned the location of enteric valve seating which is in a way important in the identification of worker castes. Besides in the specimens studied presently, P^2 is U-shaped. Additional information is that in *A. dentatus* Ahmad from Thailand, mixed segment has been observed.

As regards the zoogeographical distribution of *Amitermes*, it is cosmopolitan. In Pakistan, however, only three species occur, these are *Amitermes baluchistanicus* Akhtar, *A. belli* (Desneux) and *A. paradentatus* (Haviland). Out of these only *A. belli* (Desneux) is common with India and is widely distributed in different ecological zone.

The genus *Amitermes* presently consist of 81 species. The Ethiopian region contain the maximum number (31) of species of genus *Amitermes*. The Australian region with 27 species of genus *Amitermes* is the second region where *Amitermes* is widely represented. In spite of the greater number of species found in Ethiopia and Australia, Emerson (1955) reported that the origin of genus *Amitermes* is obscure because more primitive relatives are Indomalayan. Bouillon (1970) has pointed out that there is evidence to suggest oriental origin of sub-family Amitermitinae with its essential part of dispersion occurring at the beginning of Cretaceous. According to Bouillon, genus *Amitermes* originated in the Oriental region and entered Africa at the beginning of Cretaceous.

The present authors, however, consider the Ethiopian region, its place of origin on the basis of the maximum number of species found in that region.

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(Received: September 27, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 59-72, 1997

IMPACT OF *AGROBACTERIUM TUMEFACIENS* CO-CULTIVATION TIME AND TEMPERATURE ON T-DNA TRANSFER AND EXPRESSION IN PLANT CELLS

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Abstract: Seventeen *Agrobacterium tumefaciens* strains were used to study the effects of co-cultivation time and temperature on the transformation efficiency of these strains in callus suspension cultures and freshly excised callus pieces of *Brassica oleracea* L. Amongst these seven strains, AM1, EI1, EI2, EI3, MI2, MI3 and MI4 were used to check the effects of co-cultivation time and temperature on the transformation efficiency of these strains in explants (leaf discs, stem cuttings and root cuttings) of five different species (*Capsicum annuum* L., *Lycopersicon esculentum* Mill., *Medicago falcata* L., *Raphanus sativus* L. and *Spinacea oleracea* L.). T-DNA transfer and expression was demonstrated by auxin independent growth of calluses/explants. Results of these experiments revealed that time and temperature play an important role in the oncogenicity, virulence and transformation efficiency of the *A. tumefaciens* strains. Generally co-cultivation for a longer period (10 minutes) and at higher temperature (37°C) yielded no transformation response. Albeit the co-cultivated calluses/explants were shifted to 24°C for T-DNA transfer and expression, but co-cultivation at higher temperature caused irreversible damage. Although varied and individual responses were observed with different strains but in general co-cultivation for 3 minutes at 28°C exhibited best transformation response for majority of strains in callus suspensions, callus pieces and explants. Transformation responses were better in callus suspensions and callus co-cultivations relative to explant co-cultivations. Co-cultivation of callus and callus suspension at 24°C for 3 minutes also exhibited transformation response but to a lesser extent. The AM1, EI2 and EI3 showed better and efficient transformation response when co-cultivated for 3 minutes time interval at 28°C with callus, callus suspension and explants. These strains also showed various morphogenetic responses in callus and callus suspension co-cultivations which were not observed with other strains.

Key words: *Agrobacterium tumefaciens*, co-cultivation, time, temperature, transformation efficiency.

INTRODUCTION

Agrobacterium tumefaciens is a soil phytopathogen which causes crown gall disease on a variety of plants (Hooykaas and Schilperoort, 1992; Howard and Citovsky, 1990; Shaw *et al.*, 1991). The virulent strains of *A. tumefaciens* harbor Ti plasmid, which ranges in size from 190 to 250 Kb, that carries genes essential for crown gall tumor induction (Wabiko *et al.*, 1990; Shurvinton and Ream, 1991). The virulence (*vir*) region of Ti plasmid is required for the transfer of T-DNA from *A. tumefaciens* to plant cells where it integrate in plant genome (Hille *et al.*, 1984; Lundquist *et al.*, 1984). Three genes (*tmsA*, *tms2* and *tmr*) located on T-DNA after

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integration in the plant genome transcribe auxin and cytokinin in transformed cells and uncontrolled and unbalanced synthesis of these hormones lead to tumor production (Shaw *et al.*, 1988; Pzour and Das, 1990; Shurvinton and Ream, 1991). *A. tumefaciens* has been widely used for transformation of plant cells (Horsch *et al.*, 1985, 1988; An, 1985; An *et al.*, 1988). The transfer of T-DNA to plant cells has been demonstrated by *A. tumefaciens* co-cultivation with plant protoplast (Depicker *et al.*, 1985), callus suspension (An *et al.*, 1988) and leaf discs (Horsch *et al.*, 1988). Temperature sensitive step is associated with *vir* region induction (Tempe *et al.*, 1977; Alt-Moerbe *et al.*, 1988; Kojima, 1990; Jin *et al.*, 1993). which ultimately control the T-DNA transfer to plant cells. At higher temperature *vir* gene expression is specially inhibited (Kojima, 1990; Jin *et al.*, 1993). The VirA and VirG protein products are involved in the induction of Ti plasmid virulence operon (Shaw *et al.*, 1988; Jin *et al.*, 1990) and VirA protein is the molecule which senses temperature (Chang and Winans, 1992). Various studies have shown that *A. tumefaciens* causes tumors on plants only at temperature below 32°C and virulence gene expression is specifically inhibited at temperature above 32°C (Kojima, 1990; Jin *et al.*, 1993). Our results on transformation efficiency of different indigenous strains of *A. tumefaciens* revealed that co-cultivation time also have impact on transformation efficiency (Qazi, 1996; Qazi and Hasnain, 1996). Hence the effects of co-cultivation time and temperature on the transformation efficiency of seventeen *A. tumefaciens* strains were investigated via callus co-cultivation and explant transformation. Results of these experiments are being reported here.

MATERIALS AND METHODS

Seventeen *Agrobacterium tumefaciens* strains, AM1, CF3, CD1, EI1, EI2, EI3, Pg2, MA5, MA6, Ma4, MI2, MI3, MI4, PR1, ST3, ST4 and TC1 were used in this study. Isolation and characterization of these strains has been described elsewhere (Qazi, 1996; Qazi and Hasnain, 1996). For growth of *A. tumefaciens* strains potato agar (Gerhardt *et al.*, 1994), nutrient dextrose agar and potato dextrose agar (Lelliott and Stead, 1987) media were used. Callus of *Brassica oleracea* L. was obtained from sterilized seeds on MS medium (Murashige and Skoog, 1962) containing 2,4-D (2,4-dichlorophenoxy-acetic acid, 4 mgl^{-1}) and coconut milk (10%). The transformation of callus and callus suspension cultures was accomplished via co-cultivation technique of An *et al.* (1988). The callus and callus suspension cultures were co-cultivated with liquid cultures, adjusted to 4-5x10^8 cells ml^{-1}, of *A. tumefaciens* strains. MS media with and without auxins was used for callus cultures, supplemented with kanamycin (100 μgml^{-1}) and carbenicillin (500 μgml^{-1}) whenever required, in illuminated light (16 hours, 3 K lux) at 25±1°C. Five plants, *Capsicum annuum* L., *Lycopersicon esculentum* Mill., *Medicago falcata* L., *Raphanus sativus* L. and *Spinacea oleracea* L., were used for explant experiments. Seeds of these plants were procured from Punjab Seed Corporation. Plant seeds were sterilized in 10% commercial sodium hypochlorite for 5 minutes. Thoroughly washed disinfected seeds were grown in autoclaved Petri plates lined with double layer of filter paper, containing 5 ml of sterilized glass distilled water in illuminated light (15 hours, 3 K lux) at 25±1°C. For explants (leaf discs, stem cuttings and root cuttings) transformation experiment was carried out following Horsch *et al.* (1988). The explants were co-cultivated with liquid cultures, adjusted to 4-5x10^8 cells ml^{-1}, of *A. tumefaciens* strains. The co-cultivated explants were blot dried and

transferred to auxin free MS medium (supplemented with carbenicillin 300 μgml^{-1} and kanamycin 100 μgml^{-1}) in illuminated light (16 hours, 3 K lux) at 25±1°C. Different combinations of time (1, 3 and 10 minutes) and temperatures (24°, 28° and 37°C) were used for co-cultivation of callus suspension cultures of *B. oleracea*, freshly excised callus pieces of *B. oleracea* and explant (leaf discs, stem cuttings and root cuttings). Auxin independent growth was the criteria for positive transformation response. Observations were made daily and changes were regularly recorded.

RESULTS

Co-cultivation of Brassica oleracea callus suspension

The callus suspension of *B. oleracea* were co-cultivated with the seventeen *A. tumefaciens* strains for three time intervals (1, 3 and 10 minutes time duration) at three different temperatures (24°, 28° and 37°C). The co-cultivated callus suspension cultures (with *A. tumefaciens* strains), along with control (treated with dilution medium for same time and temperature), were initially transferred to MS medium containing 2,4-D (4 mgl^{-1}) and coconut milk (10%) supplemented with antibiotics, carbenicillin 500 $\mu g\ ml^{-1}$ and kanamycin 100 $\mu g\ ml^{-1}$. At the end of second week all the co-cultivated calluses were transferred to auxin free MS medium without antibiotics. The calluses co-cultivated for 1 minute time duration at 24°C showed no transformation response on auxin free MS medium and by the end of fourth week in these calluses signs of browning was exhibited (Table I). Similar response was observed in the callus suspension cultures co-cultivated for 1 minute time duration at 37°C *i.e.*, lack of transformation response, but these calli showed symptoms of browning by the end of second week. In some of these calli signs of necrosis was also observed during the fourth week of incubation on auxin free MS medium (Table I). The calli co-cultivated for 1 minute time duration at 28°C showed transformation and proliferation response on auxin free MS medium (Table I). Different responses were shown by the callus suspension co-cultivated with different strains. The callus suspensions co-cultivated with AM1, EI1, EI2, EI3, MI2, MI3 and MI4 gave early callus proliferation response as compared to the rest of the strains. These strains gave transformation response during the second week of incubation on auxin free MS medium. The CD1 and MA5 co-cultivated callus suspensions showed slow callus proliferation during the second week on auxin free MS medium but during the third week of transfer on auxin free medium a rapid callus proliferation was observed in both cases. The CF3, Pg2, MA6, Ma4, PR1, ST3, ST4 and TC1 showed delayed transformation response and slow callus proliferation. These strains gave callus proliferation response during the third week of transfer on auxin free MS medium, except MA6 and TC1 which showed callus proliferation response during the fourth week of incubation. The callus suspensions co-cultivated for 1 minute time duration at 28°C with AM1, EI2 and EI3 also exhibited rhizogenesis response during the third week of incubation on auxin free MS medium (Fig.1a). In AM1 co-cultivated calluses rhizogenesis was followed by caulogenesis (Fig.1c).

The callus suspensions co-cultivated for 3 minutes duration at 24°C with AM1, CD1, EI1, EI2, EI3, MA5, MI2, MI3 and MI4 showed transformation response but this transformation response was delayed and callus proliferation was rather slow as

compared to the transformation response of callus suspensions co-cultivated for 1 minute duration at 28°C and callus suspensions co-cultivated for 3 minutes duration at 28°C (Table I). The callus suspensions co-cultivated for 3 minutes duration at 24°C (with AM1, CD1, EI1, EI2, EI3, MA5, MI2, MI3 and MI4) gave transformation response during the third week of incubation on auxin free MS medium. The rest of the strains co-cultivated for 3 minutes time duration at 24°C showed no transformation response on auxin free MS medium (Table I). The callus suspensions co-cultivated with AM1, EI2 and EI3 (for 3 minutes at 24°C) also exhibited rhizogenesis during the fifth week of incubation (Fig.1b). The unbranched and hair like rhizoids were produced on the general surface of calli in a localized regions (Fig.1b). Whereas in the callus suspensions co-cultivated for 1 minute duration at 28°C rhizoids were formed on small protuberance like structures and the tip of protuberance was without any rhizoids (Fig.1a). The rhizoids produced on the protuberance were many fold greater in number (Fig.1a) than the rhizoids formed in the localized region of calli co-cultivated for 3 minutes duration at 24°C (Fig.1b). The callus suspensions co-cultivated for 3 minutes duration at 28°C showed transformation response in all the co-cultivated cases (Table I). However, different strains showed different transformation responses. The callus suspensions co-cultivated with AM1, CD1, EI1, EI2, EI3, MA5, MI2, MI3 and MI4 showed callus proliferation response during the second week of incubation on auxin free MS medium. The CD1 and MA5 co-cultivated callus suspensions showed slow callus proliferation response during the second week but in the third week the calli proliferated quickly. The rest of the strains co-cultivated for 3 minutes duration at 28°C showed transformation response during the third week of incubation, except MA6 and TC1. The MA6 and TC1 showed callus proliferation during the fourth week of incubation on auxin free MS medium. The callus suspensions co-cultivated for 3 minutes duration at 28°C showed better callus proliferation response as compared to the callus suspensions co-cultivated for 1 minute duration at 28°C. The callus suspensions co-cultivated with AM1, EI2 and EI3 (for 3 minutes at 28°C) showed rhizogenesis response similar to that shown by the callus suspensions co-cultivated for 1 minute time duration at 28°C (Fig.1a). In AM1 co-cultivated calli (for 3 minutes at 28°C) caulogenesis was also observed during the fifth week of incubation on auxin free MS medium (Fig.1c).

The callus suspensions co-cultivated for 10 minutes time duration at 24° and 37°C showed almost similar responses. In all cases no callus proliferation response on auxin free MS medium was observed (Table I) and the co-cultivated calluses become brown and necrotic during the sixth week of incubation. The callus suspensions co-cultivated with AM1, CD1, EI1, EI2, EI3, MA5, MI2, MI3 and MI4 for 10 minutes time duration at 28°C showed some callus proliferation response during the second week and third week of incubation on auxin free MS medium. But afterward growth in these calli was arrested followed by the browning of cells and ultimately necrosis was observed during the sixth week of incubation. The rest of the strains showed no transformation response and cells of callus suspensions co-cultivated with these strains ultimately become brown and necrotic. All the control callus suspensions showed no callus proliferation in any case (Table I). The control cultures showed signs of browning during the third week of incubation on auxin free MS medium. This browning response continued and ultimately necrosis started in the fourth week of incubation (Fig.1d).

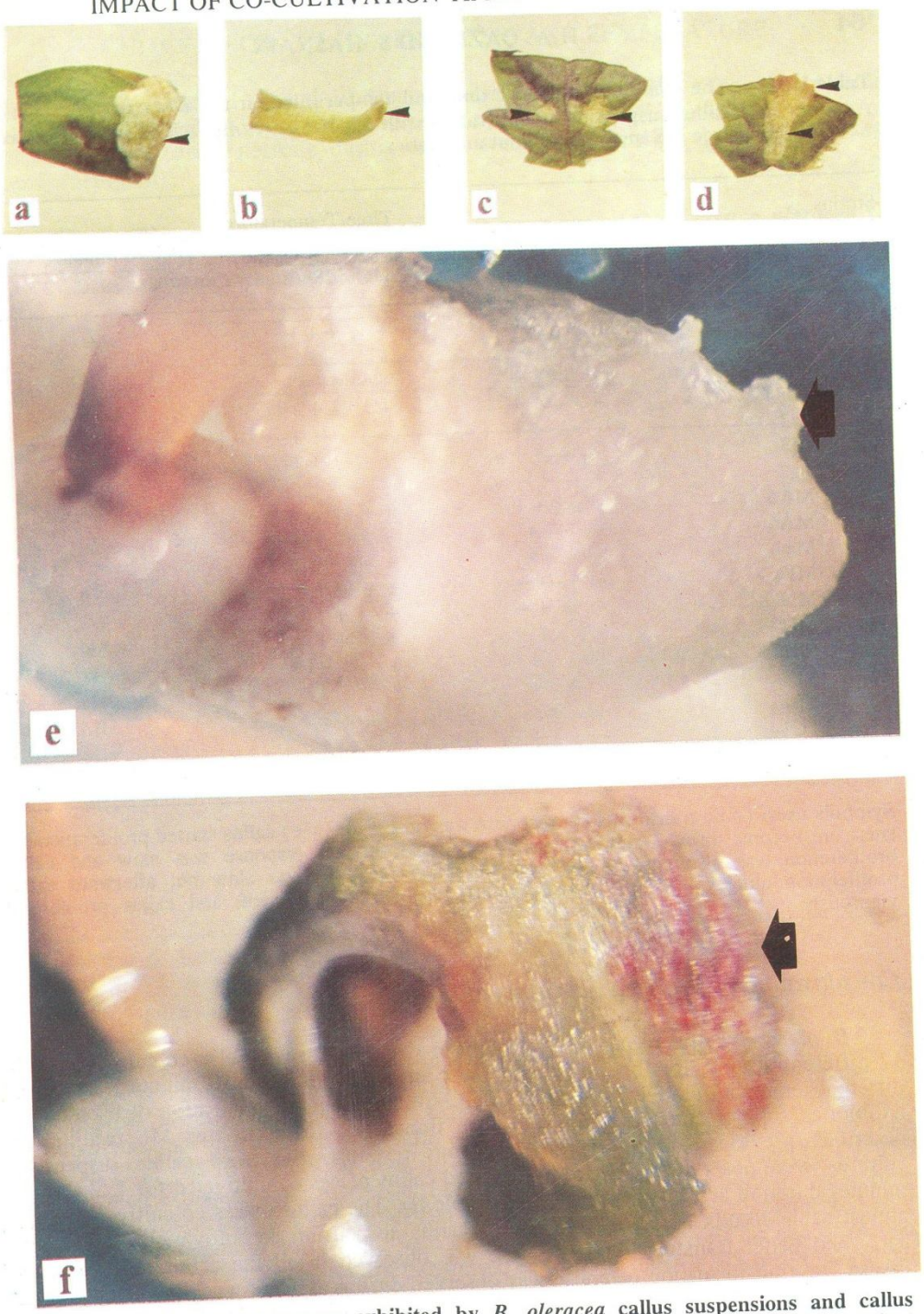

Fig. 1: Various responses exhibited by *B. oleracea* callus suspensions and callus pieces co-cultivated for three different time intervals (1, 3 and 10 minutes) at three different temperatures (24°, 28° and 37°C). a) Rhizogenesis on protuberances in a localized region; b) rhizogenesis on the general surface in a localized region; c) caulogenesis; d) control showing browning and necrogenesis.

Table I: **The combined effect of time and temperature on the *Brassica oleracea* L. callus suspension co-cultivation with selected *A. tumefaciens* strains on auxin free MS medium without antibiotics.**

Strains		Time/Temperature								
		1 Minute			3 Minutes			10 minutes		
		24°C	28°C	37°C	24°C	28°C	37°C	24°C	28°C	37°C
1.	AM1	-	++	-	+	+++	-	-	w+	-
2.	CF3	-	+	-	-	+	-	-	-	-
3.	CD1	-	++	-	+	++	-	-	w+	-
4.	EI1	-	++	-	+	+++	-	-	w+	-
5.	EI2	-	++	-	+	+++	-	-	w+	-
6.	EI3	-	++	-	+	+++	-	-	w+	-
7.	Pg2	-	+	-	-	+	-	-	-	-
8.	MA5	-	++	-	+	++	-	-	w+	-
9.	MA6	-	+	-	-	+	-	-	-	-
10.	Ma4	-	+	-	-	+	-	-	-	-
11.	MI2	-	++	-	+	+++	-	-	w+	-
12.	MI3	-	++	-	+	+++	-	-	w+	-
13.	MI4	-	++	-	+	+++	-	-	w+	-
14.	PR1	-	+	-	-	+	-	-	-	-
15.	ST3	-	+	-	-	+	-	-	-	-
16.	ST4	-	+	-	-	+	-	-	-	-
17.	TC1	-	+	-	-	+	-	-	-	-
18.	Control	-	-	-	-	-	-	-	-	-

Symbols uses: (-) no transformation response was observed; (w+) callus started proliferation but later on become brown and necrotic; (+) transformation response was slow and callus proliferation was also slow; (++) transformation response was slow but afterward callus proliferation quickly; (+++) transformation response was efficient and callus proliferated vigorously.

Co-cultivation of Brassica oleracea callus

B. oleracea callus pieces of 2 to 3 mm in size (~1 mg) were also co-cultivated with 17 different strains, for three different times intervals (1, 3 and 10 minutes) and three different temperatures (24°, 28° and 37°C), followed by the eradication of bacteria on antibiotics supplemented medium and then transferred to auxin free MS medium as described previously. Similar transformation responses, as with callus suspension cultures, were observed in the freshly excised callus pieces co-cultivated for 1, 3 and 10 minutes duration at 24°, 28° and 37°C (Table II). The callus pieces co-cultivation gave almost same results as were observed in callus suspension co-cultivations. However, early and better transformation response was observed in all the callus co-cultivation cases. The transformation response was observed during the first week of incubation on auxin free MS medium in contrast to callus suspension cultures. However, no transformation response was observed in calluses co-cultivated for 1 and 10 minutes

duration at 24°C and 1, 3 and 10 minutes time duration at 37°C (Table II). All the control calli showed no proliferation response on auxin free MS medium. The control calli started appearing necrotic during fourth week of incubation on auxin free MS medium (Fig. 1d).

Table II: The combined effect of time and temperature on the *Brassica oleracea* L. callus co-cultivation with selected *A. tumefaciens* strains on auxin free MS medium without antibiotics.

Strains		Time/Temperature								
		1 Minute			3 Minutes			10 minutes		
		24°C	28°C	37°C	24°C	28°C	37°C	24°C	28°C	37°C
1.	AM1	-	++	-	+	+++	-	-	w+	-
2.	CF3	-	+	-	-	+	-	-	-	-
3.	CD1	-	++	-	+	++	-	-	w+	-
4.	EI1	-	++	-	+	+++	-	-	w+	-
5.	EI2	-	++	-	+	+++	-	-	w+	-
6.	EI3	-	++	-	+	+++	-	-	w+	-
7.	Pg2	-	+	-	-	+	-	-	-	-
8.	MA5	-	++	-	+	++	-	-	w+	-
9.	MA6	-	+	-	-	+	-	-	-	-
10.	Ma4	-	+	-	-	+	-	-	-	-
11.	MI2	-	++	-	+	+++	-	-	w+	-
12.	MI3	-	++	-	+	+++	-	-	w+	-
13.	MI4	-	++	-	+	+++	-	-	w+	-
14.	PR1	-	+	-	-	+	-	-	-	-
15.	ST3	-	+	-	-	+	-	-	-	-
16.	ST4	-	+	-	-	+	-	-	-	-
17.	TC1	-	+	-	-	+	-	-	-	-
18.	Control	-	-	-	-	-	-	-	-	-

For details of symbols used, see Table I.

Explant co-cultivation

The effects of co-cultivation time and temperature on the transformation efficiency of seven *A. tumefaciens* strains (which gave better transformation responses in callus/callus suspension co-cultivation experiments) in the explants (leaf discs, stem cuttings and root cuttings) of five different plant species was studied for three time intervals (1, 3 and 10 minutes) at three different temperatures (24°, 28° and 37°C). The co-cultivated explants, along with control (treated with dilution medium for same time interval and temperature), were transferred to auxin free MS medium supplemented with antibiotics, carbenicillin 300 $\mu g\ ml^{-1}$ and kanamycin 100 $\mu g\ ml^{-1}$. At the end of second week the co-cultivated explants were transferred to fresh auxin free MS medium without antibiotics. The explants (leaf discs, stem cuttings and root cuttings) co-cultivated for 1, 3 and 10 minutes time interval at 24°, 28° and 37°C. All the explants of five different

plant species used for co-cultivation with *A. tumefaciens* strains for 1 and 10 minutes time duration at 24°, 28° and 37°C showed no transformation response on auxin free MS medium without antibiotics. The explants (stem cuttings and root cuttings) co-cultivated for 3 minutes time duration at 24°, 28° and 37°C also exhibited no transformation response on auxin free MS medium except for one case i.e., with AM1 co-cultivation, in which stem cutting of *Capsicum annuum* showed some callus like cells proliferation at the excised edge. The leaf discs co-cultivated for 3 minutes time interval at 37°C showed no transformation response while leaf discs co-cultivated for 3 minutes duration at 24°C gave positive transformation response only in case of *Lycopersicon esculentum* leaf discs co-cultivated with AM1, EI2 and EI3. With rest of the strains no transformation response was exhibited even in *L. esculentum*.

Out of these five plants used as explant in the co-cultivation experiments T-DNA transfer and expression was manifested only in four plants. In *C. annuum* only leaf discs co-cultivated at 28°C for 3 minutes time interval showed transformation response. The leaf discs co-cultivated with AM1, EI2 and EI3 showed transformation response during the second week of transfer on auxin free MS medium without antibiotics. The leaf discs formed callus like mass of cells at one of its excised end (Fig.2a). The stem cutting of *C. annuum* also showed some transformation response, when co-cultivated with AM1 at 28°C for 3 minutes time duration where some cells at the excised end produced small callus like mass of cells (Fig.2b). This was the only case in which stem cutting showed transformation response. All other strains were unable to show any transformation response in stem cuttings of *C. annuum*.

In *L. esculentum* only leaf discs co-cultivated for 3 minutes time duration at 24° and 28°C showed transformation response. The leaf discs co-cultivated (for 3 minutes at 24°C) with AM1, EI2 and EI3 showed transformation response during the second week of incubation on auxin free MS medium. The leaf discs formed small callus like mass of cells at localized regions (Fig.2c). The leaf discs co-cultivated with AM1, EI2, EI3, MI2, MI3 and MI4 for 3 minutes time duration at 28°C also produced callus like mass of cells at the excised edges. The leaf discs co-cultivated at 28°C produced better callus proliferation response as compared to the leaf discs co-cultivated at 24°C (Fig.1d). No transformation response was observed in any other co-cultivated explant. No transformation response was observed in co-cultivated explant of *M. falcata* with any strain. The co-cultivated explants lost their green coloration and started decaying on the auxin free MS medium in the fourth week of transfer to auxin free MS medium.

In *R. sativus* AM1 and EI3 and in *Spinacea oleracea* AM1 and EI2 co-cultivated leaf discs, at 28°C for three minutes time duration, showed transformation response on auxin free MS medium. The *R. sativus* leaf discs lost their green coloration at the end of first week of transfer on auxin free MS medium and during the second week produced callus like mass of cells from the excised edge. The callus like mass of cells were compact and white in color (Fig.2e). All other strains showed no transformation response in *R. sativus* explants. While the *S. oleracea* leaf discs did not lost their coloration on auxin free MS medium and also produced callus like mass of cells. These cells were soft and greenish in color (Fig.2f). The rest of the strains showed no positive transformation response in leaf discs as well as in all other explants of the *S. oleracea*.

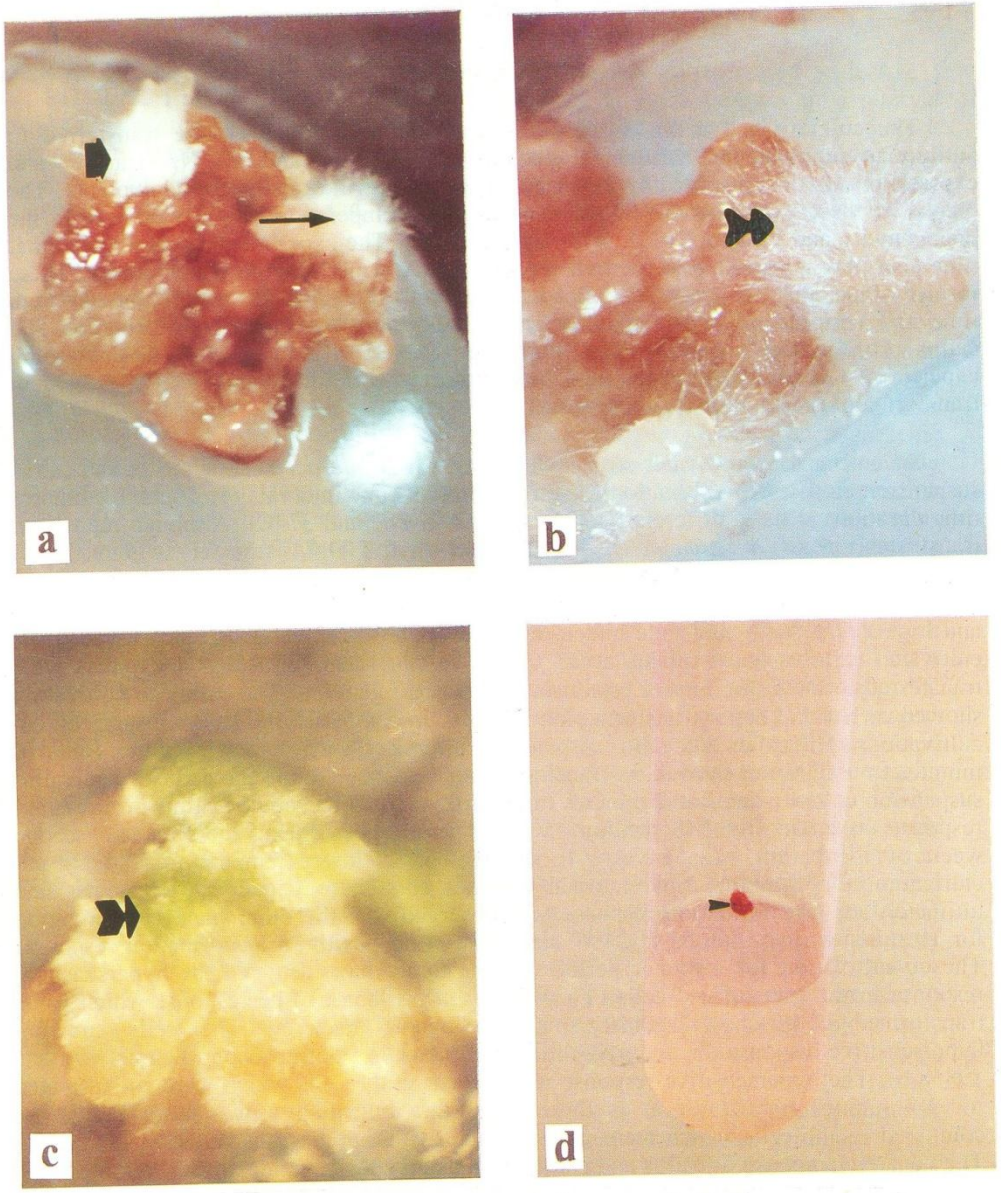

Fig. 2: Various responses exhibited by explants co-cultivated for three different time intervals (1, 3 and 10 minutes) at three different temperatures (24°, 28° and 37°C). a) Callus like mass of cells produced at the excised edge of leaf disc of *C. annuum*; b) some cells exhibiting proliferation response at the edge of stem explant of *C. annuum*; c) small tumors induced on leaf disc of *L. esculentum* co-cultivated at 24°C for 3 minutes time interval; d) somewhat larger tumors induced on leaf disc of *L. esculentum* co-cultivated at 28°C for 3 minutes time interval; e) white compact callus like mass of cells induced on *R. sativus* leaf disc; f) soft greenish callus like cells formed on *S. oleracea* leaf disc.

DISCUSSION

The co-cultivated callus suspensions, calluses and explants were transferred to antibiotics supplemented media to eradicate the bacteria and T-DNA transfer and expression was demonstrated by auxin independent growth of calluses and explants. The results of these experiments showed that time and temperature play a vital role in the oncogenicity and virulence of *A. tumefaciens* strains. The experimental data showed that time also play a crucial role for transfer of T-DNA at permissible temperatures. These results reflect that for callus co-cultivation as well as explant co-cultivation optimum time and temperature was 3 minutes and 28°C where maximum transformation and callus proliferation responses were observed (Table I, II). In explant co-cultivation experiments at the same time and temperature (3 minutes and 28°C) maximum transformation results were also achieved.

Seventeen *A. tumefaciens* strains were used for *B. oleracea* callus pieces and callus suspension cultures co-cultivation for three different time intervals (1, 3 and 10 minutes time duration) at three different temperatures (24°, 28° and 37°C). Although reports on transformation of *A. tumefaciens* demonstrate that T-DNA transfer is temperature sensitive phenomenon, and temperature above 32°C hinder *vir* gene induction, a prerequisite for transfer of T-DNA. According to Jin *et al.* (1993) at temperature 32°C and higher, the VirA molecule undergoes a reversible inactivation. Hence we also co-cultivated calluses and explants at 37°C and co-cultivated calluses and explants were transferred to 24°C for further establishment in plant genome. The callus co-cultivation showed earlier and better transformation response in comparison to callus suspension co-cultivations. The callus and callus suspension cultures co-cultivated at 24°C for 1 and 10 minutes time duration showed no transformation response. While the calluses and callus suspension co-cultivated for 3 minutes time duration at 24°C showed callus proliferation response on auxin free MS medium (without antibiotics) during the first and second week of incubation, respectively. It shows that at lower temperature (24°C) co-cultivation for 1 minute time interval was not enough to trigger the *vir* genes and ultimately no transformation response was observed. On the other hand co-cultivation for 10 minutes time interval at 24°C also showed no positive transformation response. The co-cultivation for a longer period of time might have actuated the hypersensitive response in the transformed cells (Yanofsky *et al.*, 1985a,b), the response which some transformed cells show against some pathogenic bacteria. The cells showing hypersensitive response die and save the rest of the cells from invasion (Yanofsky *et al.*, 1985a,b). The hypersensitive response was evident in the cells which were co-cultivated for 10 minutes time duration as these cells started turning brown more rapidly as compared to the cells which were co-cultivated for 1 minute time duration (at 24°C). The negative response of callus and callus suspension co-cultivations for 10 minutes time duration (at 24°C) might also be due to co-suppression factor. During co-cultivation several T-DNA copies are transferred to some cells and in some cases these T-DNA copies lead to gene inactivation (Hooykaas and Schilperoort, 1992; Matzke *et al.*, 1989). There might be a possibility that at 24°C the co-cultivation for 10 minutes time interval lead to insertion of several T-DNA copies in the transformed calluses, which ultimately inactivated the gene expression. Alternatively both phenomenon, hypersensitive response and co-suppression effect, might be responsible for the negative transformation response of the co-cultivated callus pieces and callus suspensions on

auxin free MS medium. The callus and callus suspensions co-cultivated with AM1, CD1, EI1, EI2, EI3, MA5, MI2, MI3 and MI4 at 24°C for 3 minutes time interval showed callus proliferation on auxin free MS medium during the second and third week of incubation, respectively. Here response was delayed and calluses proliferation was slow as well. In callus and callus suspension co-cultivations at 24°C for 3 minutes time rhizogenesis was also delayed and rhizoids were produced on general surface of the proliferating callus (Fig.1b).

The callus suspensions and calluses co-cultivated at 28°C for 1 and 3 minutes time duration gave better transformation response as compared with callus transformation response observed at 24°C (for 3 minutes time interval). This response shows that 1 and 3 minutes co-cultivation time at 28°C were suitable for *vir* region induction and T-DNA transfer in the co-cultivated cells. The rhizogenic response was more vigorous at this temperature and with AM1 co-cultivation at this temperature pronounced caulogenic response was also observed. It seems that this time and temperature are not only optimum for *vir* gene induction, but they might also influence subsequent transformation mechanism/s. At this time and temperature the co-cultivation of callus and callus suspension might activate insertion elements present on T-DNA. The insertion elements play a role in the abnormal production of auxin and cytokinin (Ponsonnet *et al.*, 1995). On the other hand the flanking DNA and chromatin structure also effect the expression of T-DNA genes in different transformed cells (Horsch *et al.*, 1988). Certain mutations in the transferred T-DNA also effect the tumor morphology of the transformed cells (Gheysen *et al.*, 1985). Mutations in the 'shooty' locus (*shi* or *tms*) produced abnormal shoot formation in the transformed cells. Whereas mutations in the 'rooty' locus (*roi* or *tmr*) caused abnormal development of roots instead of production of undifferentiated mass of cells. Unusual larger tumor formation as compared to normal tumor induction is caused by the mutation in the morphogenetic locus, *tml* (Grierson and Covey, 1988). However at 28°C a longer period of co-cultivation (10 minutes) initially showed some proliferation response on auxin free MS medium (Table I, II) but the proliferation response was accompanied by the browning of the cells, which dominated the callus proliferation. These observations demonstrate that co-cultivation for a longer period of time stir the hypersensitive response (Yanofsky *et al.*, 1985a,b) in the transformed cells (Table I, II). CF3, MA6, Ma4, PR1, ST3, ST4 and TC1 gave no transformation response when co-cultivated for 10 minutes time interval at 28°C. This might be due to more pronounced hypersensitive response of the co-cultivated cells (Yanofsky *et al.*, 1985a,b) and cells could not be survived after co-cultivation.

The callus suspensions and calluses co-cultivated for 1, 3 and 10 minutes duration at 37°C gave no transformation response. At higher temperature virulence gene expression is specially inhibited (Jin *et al.*, 1993; Kojima, 1990). Chang and Winans (1992) reported that the molecule which senses temperature is VirA protein. *In vitro* phosphorylation experiments clearly demonstrated that the function of VirA protein, autophosphorylation and phosphate transfer to VirG, are inhibited at higher temperatures (Jin *et al.*, 1993). Hence at 37°C temperature inhibition in *A. tumefaciens vir* gene induction resulted in negative transformation response of the co-cultivated calluses. These results reflect that even co-cultivation for 1 minute at 37°C inhibit T-DNA transfer or subsequent steps in transformation. Although treated calluses were shifted to 24°C but damage at 37°C was irreversible. These results are contradictory to the report

of Jin *et al.* (1993).

In explant co-cultivation experiments similar responses, as in callus/callus suspensions, were observed. The higher temperature gave no transformation response due to inhibition of *vir* genes activities. However, lower temperature (24°C) also gave, almost, no transformation response except with AM1, EI2 and EI3 strains where some transformation response was observed. These strains gave transformation response in *L. esculentum* leaf discs co-cultivated for 3 minutes time interval at 24°C. These results suggest that either plant become resistant to infection by *A. tumefaciens* at lower temperature (24°C) or some other factors might be involved in the negative transformation response. The explants, stem cuttings (except one case) and root cuttings showed no transformation response. The only transformation response was observed in the stem cutting of *C. annuum* when co-cultivated with AM1 where the response was also not very pronounced (Fig.1b). The cells of these explants (stem and root cuttings) might be more resistant to *A. tumefaciens* strains. The leaf discs co-cultivated at 28°C for 3 minutes exhibited better transformation response as in case of callus/callus suspension co-cultivations.

These results clearly demonstrated that virulence of *A. tumefaciens* is also dependent on time and temperature. At higher temperature *A. tumefaciens* virulence gene expression is inhibited and ultimate result is negative transformation response. Jin *et al.* (1993) suggested that at higher temperature the plant become more resistant to infection by *A. tumefaciens* and/or functions of some *vir* gene products are lost. It is not only the inability of the *A. tumefaciens* but other plant factors also play role in the negative transformation response (Jin *et al.*, 1993). Another possibility is that the plant factor required for *A. tumefaciens* transformation may be temperature sensitive. The higher (37°C) and lower (24°C) temperatures might shut down the synthesis of proteins required for *A. tumefaciens* attachment or transformation. Co-cultivation even for 1 minute at higher temperature caused irreversible damage. The three strains AM1, EI2 and EI3 showed efficient, better and maximum transformation responses in all the different time intervals and temperatures (except 37°C) used for co-cultivation of callus and callus suspensions. These strains also showed transformation response in explant (leaf discs) at 28°C as well as 24°C exhibiting wider temperature tolerance range for *vir* gene induction. These results reflect that these strains efficiently transfer the T-DNA at both the temperatures. The VirA protein of these strains might work equally well at 28°C and 24°C as compared to the rest of strains. A thorough knowledge of Ti plasmids, present in these strains, is required to know the actual mechanism involved.

Acknowledgement

UGC is acknowledged for the award of scholarship to S.H.A. Qazi.

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(Received February 10, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 73-84, 1997

ON BIOLOGY OF HOUBARA BUSTARD (*CHLAMYDOTIS UNDULATA MACQUEENII*) IN BALOCHISTAN, PAKISTAN: A PRELIMINARY STUDY ON MIGRATION

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Abstract: Data on density, previous population levels, presence of occasional and regular flocks and direction of bird movement, collected during early and late winters for different broad localities suggest that Houbara gradually moves through diffused routes into and out of Balochistan (Pakistan), keeping a predominantly north-south direction during autumn and south-north direction during spring. These movements are largely controlled by temperature, but latitude gradient is more important in forcing autumn movements, and spring movements are more influenced by altitudes. Steep rocky hills act as strong barriers, while feeding grounds and human disturbance have little role in controlling such movements. Evidences suggest that bird movements are more controlled by external environmental factors than the internal biological clock of the species. The species depend more on walking for migratory movements, though may resort to different stretches of low flights. Overlapping of breeding and non-breeding ranges of the race suggest that mass scale movement occurring during autumn and spring does not strictly fall under the definition of migration, and can be regarded as population adjustments along its distribution range during summer and winter to fight unfavourable temperatures and associated biotic or abiotic factors.

Key words: Population movements, migratory routes, mode, migratory status, physical factors.

INTRODUCTION

Asian race (*Chlamydotis undulata macqueenii*), unlike other two races of Houbara mainly comprises of a migratory flock of birds. It largely breeds in relatively colder northern latitudes (Central Asia, Afghanistan and northern Iran) during summer and migrates into southern latitudes to winter in southern Iran, Pakistan, India and Gulf States (Ali and Ripley, 1983; Cramp and Simmons, 1980). This mass scale migration is a unique phenomenon in bustards. Though, there has been a general interest of the workers in this fascinating phenomenon, yet information on migratory routes, mode and behaviour remains sketchy. Present study was undertaken with the view that Balochistan, receiving the major chunk of the migratory flock during winter (Mian, 1983, 1984, 1988), can work as suitable tract to provide some basic information on this phenomenon.

0079-8045/97/0001-0071 $ 03.00/0

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MATERIALS AND METHODS

Physical tours of favourable bustard tracts of Balochistan (Zhob, Punjpai, Chagai, Kharan, Sibi-Kacchi, Khuzdar, Punjgur, Makran, Lasbella) were conducted during early (September) and late (March) winter between 1984 and 1987 and absolute Houbara population density recorded by transect method (Mian, 1997).

Foot tracks of Houbara were searched in different localities for 15-30 minutes by different number of workers to develop relative density indices on previous population levels. Relative density estimates were compared with absolute population density figures as indicator of population movements. Field records were maintained on general orientation of Houbara movements, gathered from direct observations on the birds and/or through tracing foot tracks. These records were suitably organised to evolve a general direction of the bird movement for different areas. Information on general topography, size and orientation of the valleys and nearby mountains, temperature, vegetative state, human settlements and soil was also recorded.

General inferences were drawn from the data on population levels, general direction of the bird movements and indices of previous populations, as seen in the background of the various biotic and abiotic factors. These inferences were resolved to evolve broad patterns on routes and mode of migration. Activity and behaviour were traced through direct field observations on selected birds and/or through following their foot tracks. Information on the dates of arrival of Houbara and relative population levels during different parts of the wintering period in different broad localities was collected through the hunters and prominents of the area. This information was confirmed through the transect data collected on density estimates and used to develop general inferences on migration biology.

RESULTS

A synopsis of information on the status of the wintering population of Houbara, during different calendar months in different broad localities of Balochistan (Fig. 1) suggest that during early winter occasional birds appeared in early August at 31°N (Zhob), late August at 29.5° (Chagai), early September at 28° (Kharan) and 28.5° (Sibi-Kacchi), mid October at 27° (Rakshan) and late November or early December at 25.5° (Makran and Lasbella). A similar pattern was generally followed in early winter appearance of regular flock in different areas located at decreasing latitudes. The appearance of occasional (late November) and regular (late December) wintering flock in Khuzdar (28°N) did not fit into this basic pattern. No regular pattern was followed with regard to altitude gradient.

Late winter disappearance of regular and occasional populations (Fig. 1) did not follow a latitude associated pattern. It obeyed the altitude gradient. The populations disappeared earlier (January-February) in southern lowlands (sea level: Lasbella, Makran) and eastern lowlands (sea level: Sibi and Kacchi). The occasional populations persisted till early/mid April in highland valleys of Kharan (800 m) and Chagai (900 m) and till late April in Rakshan (1,000 m). In northern highlands (1,400 m), late winter

population build up started in mid February and continued till mid March, while some occasional birds persisted even till late April. The population disappeared rather earlier from Khuzdar (900 m, mid February) than demanded by altitude.

Figure 2 presents average density of mid September population at different geographical locations along with general direction of bird movement and orientation of valleys and mountains. A general look at the figures suggests that an appreciable population of Houbara was present in eastern and western Chagai, western and central Kharan, Sibi-Kacchi tract (eastern Balochistan), Pishin and Patao (Zhob). No significant population was present in favourable tracts of eastern and southern Kharan, Khuzdar, Rakshan, Makran and Lasbella.

During September, the birds/foot tracks were frequently spotted in depressions and dried water courses in eastern and western Chagai and western Kharan. Individuals or groups of two/three side by side casually walking birds were frequently observed to maintain almost straight walking lines for a considerable distance with a constant tendency of a consistent southward direction of their movement. In Chagai, population was very scattered in the northern parts (as represented by low densities), yet it gradually concentrated in southern parts. The maximum concentration appeared in south-western parts, where steep and rocky Raskoh range is gradually replaced by undulating sand dunes. Equally high concentrations were also observed in north-western Kharan with a southward population movement. In eastern Balochistan, the bird were evenly scattered in suitable habitats and no uniform pattern of bird movement was followed. In Zhob, foot prints indicated a westward movement, running parallel to the orientation of two mountains bounding rather narrow valley of Patao. Central Kharan exhibited low population densities with a predominantly eastward movement of the birds along the Raskoh Range.

Presentation of late winter densities (Fig. 3) generally suggests a distribution similar to that of early winter. However, comparatively higher populations were present in eastern Kharan, central Chagai, Maslakh and Rakshan valleys, while limited populations were spotted in Sibi-Kacchi, Kapir and Khuzdar. No significant population was recorded from Makran and Lasbella. The orientation of foot tracks and/or birds did not yield a consistent direction of movement in any locality.

Relative density figures evolved for different localities from track search data during early and late winter did not agree with population levels suggested through transect data for the respective area. This was especially true for early winter data regarding northern and central highlands where a higher previous population was always indicated. Reports carried by shepherds, nomads and/or hunters could also not be confirmed by the transect data.

Activity of Houbara traced through direct observations and/or through following the foot prints during September survey, suggested that the bird frequently moves towards *Anabasis* sp., *Ziziphus* sp., *Gaillonia aucheri* and other plant species, which bore fleshy shoots, fruits and/or leaves.

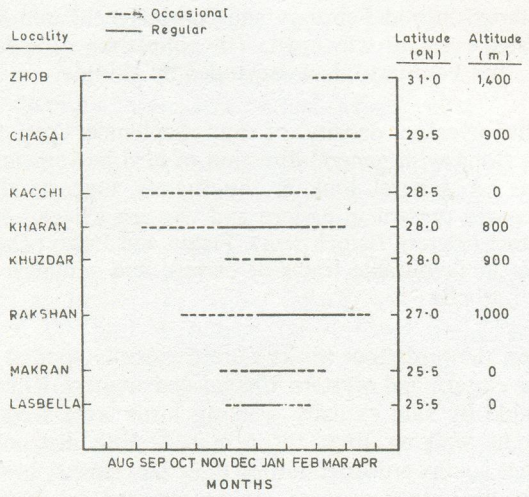

Fig. 1: **Periods of presence of regular/occasional populations of Houbara in different broad localities of Balochistan with relevant information on altitude and latitude.**

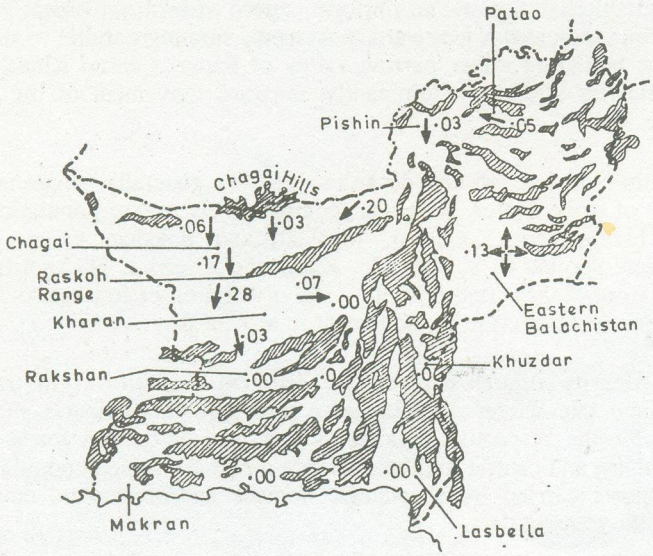

Fig. 2: **Line sketch of Balochistan showing population density of early wintering Houbara at different locations. Arrows represent direction of more frequently met flock of birds.**

DISCUSSION

Conventional and more reliable methods, like, ringing, banding or colour marking, usually employed in studies on bird migration, are rather difficult and of limited value when employed for Houbara. This is because of a wide distribution, cursorial mode, limited population and camouflaging plumage in this bird species, all making its casual detection as difficult. No safe practical trapping technique, generally required for such studies, is available in this species (Taylor, 1985, 1985a). Further, exploitation of such techniques for Houbara also demands an international co-operation, involving a battery of careful field workers scattered widely along its distribution range (Central Asian States, Afghanistan, Pakistan, India, Iran). Technique involving radio tracking of selected birds is expensive and requires wider network of trained researchers. Satellite tracking is also expensive and can only be exploited on a few birds hence is of a limited value. Under these circumstances, unconventional methods, like, exploiting population levels, period of stay, direction of the moving birds and behavioural studies used in conjunction with physico-biotic conditions can be the best possible alternative. This can give an entering wedge into studies on migration biology with limited financial implications.

Part of the year exploited for present studies on immigrating (September) and emigrating (March) flock can be justified. Available records on Houbara (Iran: Scott, 1975; Mansoori, 1974, 1985; Razdan and Mansoori, 1989; Cholistan: Mirza, 1985; Sindh: Surahio, 1981; Balochistan: Mian and Surahio, 1983; Mian, 1984, 1988) suggest that though some sporadic birds can be seen during August-September, yet the major part of the flock appears in different wintering grounds during October-November. These reports also suggest that though the bird populations appreciably decrease in different wintering grounds by February, yet the evacuation is not complete till March. Results of our studies on population fluctuations (Mian, 1997a) also suggest presence of appreciable populations of Houbara in different tracts between October-November and February-March.

Routes

Depending upon our results, tentative routes adopted by autumn migrating flock have been proposed (Fig. 4). Almost same routes appear to be adopted by back migrating flock during spring with slight adjustments under the available physico-biotic conditions. These migratory routes generally agree with those suggested previously (Mian, 1984, 1988), based upon information gathered from local hunters/populace through questionnaires.

Our results suggest that the species adopt a general north-south orientation during spring migration with slight variation forced under location of barriers to Houbara movement. This indicates that the flock of Houbara enters Balochistan from/through Afghanistan. These findings go in a good degree of conformity with reports on Pakistan (Roberts, 1985) and Iran (Mansoori, 1974, 1985; Scott, 1975; Razdan and Mansoori, 1989). The movement of the populations from Iran into Balochistan, as suggested by some reports (Anonymous, 1972; Karim and Hasan, 1983), could not be confirmed.

Very diffused migratory routes adopted by Houbara, as suggested by the present results, have been reported previously (Mian and Surahio, 1983; Mian, 1984, 1988, Goriup, 1983). At places, however, the migrating flocks pass through narrow valleys where migratory routes appear to be slightly defined. Migratory flock has to pass through rather defined routes in western Chagai, Zhob, Punjpai and Rakshan. Well-defined migratory routes suggested for this species (Roberts and Savage, 1971; Anonymous, 1972; Karim and Hasan, 1983) can only be attributed to limitations of previous observations to more accessible parts of the Province.

Adoption of diffused migratory routes can save species from probable hunting losses (Mian, 1989). The major part of the population of this race funnels through the western flanks of Chagai. The species, in the recent years, have started meeting a heavy hunting pressure both in early (Chagai) and late (Kharan) winters through the hands of falconers in this migratory funnel zone (Mian, unreported data).

Movements

Present results suggest that Houbara keeps a slow and gradual penetrance towards southern latitudes during autumn and towards northern ones during spring. Such a movement continues in the major parts of Balochistan and for the major parts of the wintering season, stable populations appearing between December and January. The stable populations with little direction oriented movements appear for an extended period (early September to mid February) in central lowlands (Sibi-Kacchi), located at an almost sea level. On the basis of the presently available results the migratory movements appear to be influenced by:

i. Steep rocky hills

Absence of Houbara in central and eastern Kharan and presence of appreciable populations in adjacent parts of Chagai during September suggest that east-west oriented range (Raskoh) of steep mountains act as a physical barrier to direct southward population movements. A similar explanation can be afforded for population build up in Kharan and late appearance of populations in central and eastern Chagai during March. Variation in the direction of autumn migrating flock in Zhob, Punjpai, eastern Chagai and central Kharan can also be explained on identical logic. The steep mountains with rocky background are believed to constitute and unfavourable habitat for Houbara (Ali and Ripley, 1983; Cramp and Simmons, 1980; Mian and Surahio, 1983; Mian, 1984, 1988).

ii. Latitude temperature complex

Late arrival of autumn migrants in southern latitudes seems fairly explained on physical distance of these tracts from summering grounds. Such a pattern has been indirectly indicated by suggesting the appearance of autumn migrants in early September in northern (Cholistan, Mirza, 1985) and in mid October in southern (central Sindh, Surahio, 1981) latitudes. The latitude (distance) logic does not conveniently explain pattern of population build up in Khuzdar, where probably other physico-biotic factors control late arrival of the bird flock.

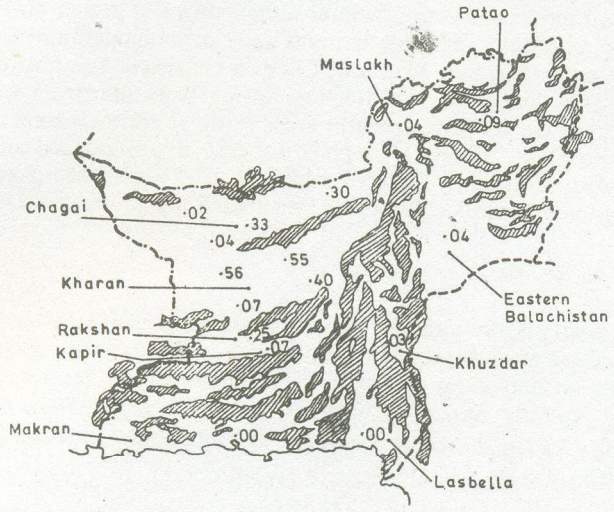

Fig. 3: Line sketch of Balochistan showing population density of late wintering Houbara at different locations.

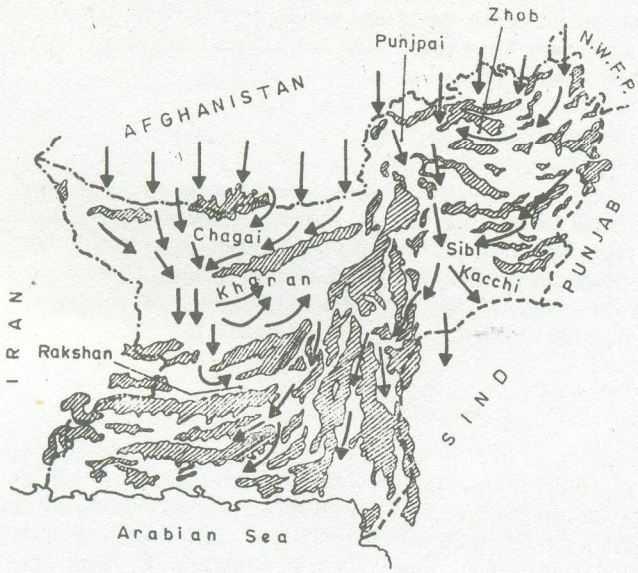

Fig. 4: Line sketch of Balochistan showing proposed migratory routes adopted by autumn migrating flock of Houbara. The hatched areas represent approximate location of the mountains.

An almost complete absence of bird population, for the major part of winter in northern highlands and during mid winter in Chagai suggest the influence of temperature in forcing southward movements. Low winter temperatures at higher altitude (>900 m) may cause autumn migration. Altitude-latitude associated temperature gradient, in the western flanks (Kharan, Rakshan, Makran), forces a consistent southward movement of the flock. Early disappearance of bird flock in spring from the tracts located at lower altitudes (Sibi-Kacchi) and its persistence in areas lying at almost similar latitude but at higher altitudes (Rakshan, Kharan, Chagai) suggest a pronounced effect of rising temperature on spring migration. Gradual withdrawal of population during late winter under increasing altitude appears explainable under such logic.

iii. Feeding grounds

General observations on migratory flock suggesting that Houbara grazes during migratory movements may indicate significance of feeding grounds in such movements. Our results provide no indication of a special preference of autumn migrating flock for favourable feeding grounds. Spring movements, however, appear to be more influenced by feeding grounds, as suggested by build up of populations in areas having suitable feeding conditions.

iv. Disturbance

Human disturbances can potentially influence migratory movements in this wary species. However, such disturbances can though affect local movements, yet are not sufficiently potent to influence migratory movements. Severe disturbances coming from falconers in Chagai, year after year, have not affected migratory movements through a gateway located in western Chagai.

v. Biological clock

Influence of internal biological clock in controlling time of onset of migration is hard to be separated from other factors. However, there are evidences to suggest that appearance of migrating flock in a specific area varies considerably between the years in response to temperature, rainfall and vegetative condition. These observations suggest a greater control of external physico-biotic conditions on migratory movements as compared with internal biological clock of the species/race.

Mode

Migratory activities in Houbara have been generally visualised believing that flight is adopted for reaching summering or wintering grounds. However, no report suggests sighting of flock on long migratory flight. This has resulted in a considerable confusion. Different workers have recorded different sizes of migrating flock, part of day exploited for migration and approximate dates when a migratory flock reaches or leaves some area. Size of migrating flock appearing in literature (Mian and Surahio, 1983; Mian, 1984, 1988; Alekseev, 1980) merely indicates number of birds seen together. Difficulties have frequently been encountered to offer valid explanation for certain observations on biology of the species, like mother embarking upon migratory

movements with very young chicks, still unable to take longer flight (Mian, 1985); the possibility of this basically cursorial bird getting exhausted during longer stretches of flight (Siddiqi, 1972) and strictly avoiding steep and rocky mountains even during migration (Mian, 1984, 1988).

Alternative hypothesis may suggest that this species mainly depends upon walking for its migratory movements. Observations on persistent walking lines and very frequent appearance of foot tracks in southern parts of western Chagai appearing to pass over the undulating sand dunes (preferred habitat), located towards the western extremity of the steep rocky Raskoh Range (not preferred as habitat) may provide some direct support to the hypothesis. This hypothesis can conveniently explain the following aspects of Houbara biology:

a) There are persistent reports from both summering (Alekseev, 1980) and wintering (Mian and Surahio, 1983, Mian, 1984, 1988; Mirza, 1985) grounds suggesting that Houbara during its migration stops for some days in different areas to exploit the available food. The bird is believed to stop every 1-3 km for grazing (Mian and Surahio, 1983). b). The species is basically cursorial. It can efficiently take short flights; but is not adapted for longer flights (Roberts and Savage, 1971). There is a possibility of this bird getting exhausted during longer flights (Siddiqi, 1972) required for bird migration. c). There is a gradual build up of populations in both summering and wintering grounds, slowly penetrating into deeper parts. The present study suggests that though population starts appearing in northern latitudes in August, yet occasional birds do not appear before early November in extreme southern latitudes (taking three months to reach a crow flight distance of about 500-600 km). Similarly occasional birds appear in southern parts of Central Asia in March, while these are not common in valleys of the Altai Mountains till late April or early May (Cramp and Simmons, 1980). d). Difficulties have been consistently faced in recording the time of initiation of migration. Ponomareva (1979) and Alekseev (1980) suggest that migration from summering grounds (Kyzylkum) starts in early August and lasts till November. Similar inconsistency is present in different reports suggesting the dates of initiation of spring migration from the wintering grounds (Mirza, 1985; Mian, 1984, 1988). The inconsistency appearing in such data can be attributed to the difficulties faced in recording the initiation of migratory movements when bird adopts walking for such movements. Flying flocks/birds can be easily recorded even by a casual observer and hence a consistency is expected in the reported dates of initiation of the migratory activity.

Our present hypothesis suggests that the species basically rely upon the cursorial mode to move between summering and wintering grounds. This does not exclude the possibility of occasional short or slightly longer low flights (suggested by limited data on satellite tracking. Launay, 1996, NARC, Abu Dhubai, personal communication) in response to some immediate demands. Such flights constitute a regular part of general habit of this species. The presently available information suggests that the bird keeps on moving casually, picking food but maintains a consistent orientation during migratory movements. The species has been reported to show extensive movements even during its regular foraging (Cramp and Simmons, 1980). Comparatively long legs with upright body posture suit this habit. Such a migratory behaviour is not expected to cause

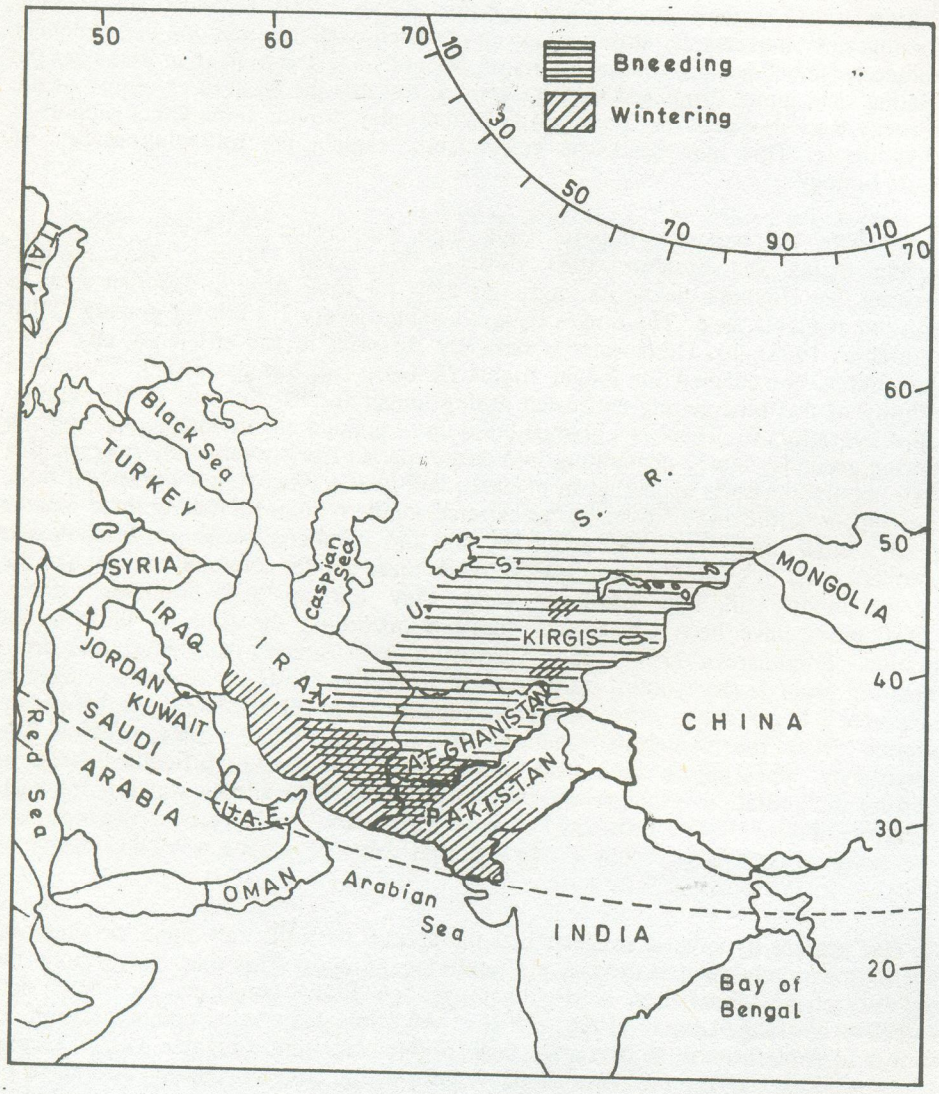

Fig. 5: **Distribution ranges of breeding and non-breeding populations of the Asian race of Houbara.**

physiological stress on the bird and can save the species from the excessive migratory losses.

Migratory status

Migration is defined as a periodic round trip of a population of a species/race between spatially isolated breeding and non-breeding grounds (Baker, 1978; Orr, 1970). Migratory movements in Houbara partially fit this definition, i.e., moving during regular seasons from northern to southern latitudes and vice versa, vacating some areas during summer and others during winter. However, available information on summering and wintering ranges suggests that breeding and non-breeding tracts of this species/race are overlapping (Fig.5) Under such conditions mass scale movements in this bird species occurring during autumn and spring does not fall under strict definition of migratory movements.

It appears that the Asian race of Houbara has a distribution range running from the Altati Mountains, through central Asia, Afghanistan, Iran, Iraq, Gulf States, Arabian States, Jordan, Israel, Pakistan, to parts of India. Population of this race is absent from parts of central Asia, Afghanistan and northern Iran, during winter and from southern and eastern parts of Balochistan, P[illegible]ab, Sindh and India during summer. Buffer areas exist where the populations persist throughout the year. This race appears to have a wider distribution range during breeding season (summer) as demanded under biological requirements. Birds concentrate in a comparatively narrow distribution range during non-breeding season (winter) in response to availability of favourable temperature and feeding grounds. Thus, such movements can be regarded as population adjustments in an attempt to extract optimal benefits out of available physico-biotic variation, rather than as strict migration. Such a status equips the species/race to harvest the biotic benefits of migratory behaviour (finding optimal survival conditions under seasonal odds, saving over-exploitation of restricted tracts) as also saves it from the migratory stresses.

Acknowledgements

This study was undertaken with a partial support from WWF-Pakistan and in active collaboration with Balochistan Forest Department. Thanks are due to a number of hunters, field guides and members of Forest department for their help and support during field studies.

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(Received: July 10, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 85-90, 1997

METABOLITES OF *DINOTHROMBIUM TINCTORIUM* (BIR BAHUTY), AS AN APHRODISIAC SUBSTANCE

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Abstract: Metabolites of *Dinothrombium tinctorium* (Bir bahuty) were studied for their aphrodisiac potential, which deliberates upon the fertility enhancement potential of the rice mixed with metabolites of the mites, as administered in the Unani system of medicine. The results indicated significant increase in sperm count, weight of gonads and accessory glands, as well as the diameter of seminiferous tubules, spermatogonium, primary and secondary spermatocytes, spermatid and spermatozoa. The testosterone level in serum and the testicular tissue also increased significantly. The chemical pathology of the treated group was found to be within normal range.

Key words: *Dinothrombium tinctorium*, metabolites, aphrodisiac.

INTRODUCTION

Over 70-80 percent of population in the third world countries live in the rural areas and are often familiar with, and have faith in, the folk-lore and traditional systems of medicine prevailing in their areas. Fairly large number of medicinal plants (herbs), minerals and animal products are reported to have been used for fertility regulation in human beings.

In connection with our efforts to develop indigenous resources and establish their efficacy (through laboratory trials) for fertility regulation of male, we have evaluated a number of prescriptions and indigenous materials, aiming towards isolation of some natural material with potentials to regulate male fertility. *Dinothrombhium tinctorium* (Bir bahuty) is known in Unani System of Medicine for having aphrodisiac effect in males (Kabir, 1937; Board of Ayurvedic System of Medicine, 1971) and is administered both as total animal powder as well as ground rice mixed with the metabolic products of the mites.

Earlier studies in our laboratory have examined the aphrodisiac nature of the total animal powder of these mites (Subhan *et al.*, 1988, 1989a, 1989b, 1990a, 1990b, Subhan and Khan, 1991; Subhan, 1995). The hormonal profile analysis of the total animal powder of the mites has revealed significant levels of steroidal hormones (Subhan

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et al., 1995), and the total animal powder was also found to be rich in lipids, the main precursor of these androgenic hormones (Subhan and Tahir, 1996).

The present study is aimed at exploring the aphrodisiac potential of the metabolities of mites, as Shideler *et al.* (1993) have demonstrated that animal metabolites contain a reasonable amount of steroids.

MATERIALS AND METHODS

Animal trial

Adult male rats (Sprague dawley strain), were used in the study. The animals were divided into two groups as control and treated, each group containing 20 animals. Live Bir bahuty were placed on rice to collect their metabolites on rice. After thorough mixing, a dose of 100 mg finely ground rice and metabolite mixture was given orally to the treated animals for 8 weeks, in order to evaluate the effect on spermatogenesis. Food and water was available *ad libitum*. The parameters which were observed included semen analysis, weight of accessory glands, histological studies and testosterone level in serum and interstitial testosterone concentration.

Sperm count

Semen collected through electric ejaculation (Moore and Gallagham, 1930) was analysed for sperm concentration with the help of Mackler chamber. The sperms were counted from the whole cauda, which was put in a small test tube in 1 ml ptyroid's solution and cut with scissors into small pieces and then another 1 ml of ptyroid's solution was mixed, shaken and was placed for some time. The cauda pieces were removed with the forceps and the suspension was centrifuged for 30 minutes at 600 rpm. The pellet was washed with ptyroid's solution and then 2 ml solution was mixed and counted with a Neubauer counting chamber. The number of sperms were obtained by multiplying the sperm count in 10 squares of the grid or total number of sperms were counted (Parkashi *et al.*, 1985).

Histological study

One testis of each animal was subjected to histological process for observation of different stage of spermatogenesis, seminiferous tubules, interstitial cells and sertoli cells.

Blood chemistry

Serum of the experimental animals was subjected to estimation of testosterone with RIA and biochemical test by the established methods for clinical chemistry.

RESULTS AND DISCUSSION

The treatment of rice containing metabolites of mites resulted in a significant increase in weight of the gonads and accessory gonads. As testosterone levels both in serum and testes (Table I), diameter of the histological sections of the testis (Table II) and higher sperm count/cauda epididymis (Table III). Blood chemistry analysis of the treated and control groups, except for serum and testes testosterone levels, show no significant differences (Table IV).

Table I: Effect of metabolites of mites on body weight, gonads weight and accessory gonads.

	Treated	Control
Initial weight of animals (g)	149.00±03.45[a]	153.00±02.550
Final weight of animals (g)	267.20±11.11	281.80±03.810
Weight of testes (g)	2.90±00.08	2.82±00.110
Weight of seminal vesicles (g)	1.95±00.09	1.13±00.090
Weight of prostrate (g)	0.91±00.05	0.48±00.050
Weight of epididymis (g)	0.82±00.04	0.38±00.010

[a] Mean ± SEM

Table II: Effect of metabolites of mites on morphometric observation of testicular section, with reference to diameter.

Parameters	Diamter (μm)	
	Treated	Control
Seminiferous tubules	196.24±2.71[a]	186.04±2.86
Leydig Cells	5.06±0.29	4.34±0.19
Sertoli Cells	7.29±0.22	6.35±0.32
Spermatogonia	5.07±0.10	4.32±0.14
Primary Spermatocytes	7.92±0.09	7.10±0.19
Secondary Spermatocytes	4.87±0.11	4.31±0.23
Spermatids	4.70±0.16	4.10±0.14
Spermatozoa	0.51±0.02	0.42±0.03

[a] Mean ± SEM

Table III: **Effect of metabolites of mites on sperm motility and sperm count.**

	Treated	Control
Sperm count (Mx10^6/cauda epididymus)	748.0±24.14	604.0±26.65
Motility		
Motile (%)	85.8±2.29	84.2±1.68
Non-Motile (%)	14.2±2.29	15.8±1.68
Motile/non-motile sperms	6.84	5.31

Table IV: **Effect of metabolites of mites on the blood chemistry and gonads testosterone concentration.**

	Treated	Control
Protein (mg/dl)	8.23±0.11[a]	8.17±0.27
Cholesterol (mg/dl)	73.08±4.57	76.77±3.22
Lipids (mg/dl)	130.40±6.89	134.97±5.79
AP (U/l)	96.70±8.96	94.40±9.12
SGOT (U/l)	25.90±1.04	26.12±0.90
SGPT (U/l)	23.70±1.08	22.43±1.14
Bilirubin (mg/dl)	0.38±0.01	0.36±0.01
Creatinine phosphate (mg/dl)	0.73±0.01	0.71±0.03
Glucose (mg/dl)	82.42±2.76	81.25±1.93
Urea (mg/dl)	28.30±1.65	26.35±1.48
Serum testosterone (ng/ml)	0.93±0.20	0.61±0.16
Testes testosterone (ng/mg)	0.20±0.04	0.11±0.004

[a] Mean ± SEM

Abbreviations: AP, alkaline phosphatase; SGOT, serum glutamate oxaloacetate transaminase; SGPT, serum glutamate pyruvate transaminase.

The observations are in agreement with the reports of Kabir (1937) and the Board of Unani and Ayurvedic System of Medicine (1971) in having aphrodisiac effect of the rice having metabolites of mites.

The histological observation of the testicular section is suggestive of the fact that the metabolites of mites do posses some potent agent which has enhanced the process of spermatogenesis. The effect at the moment cannot be characterized due to the androgen or pituitary gonadotrophins, or due to nutritional factors. The normal level of blood chemistry, however, revealed a normal picture of the treated group.

The mode of action of the metabolites of mites, at hypothalamus, pituitary/or gonadal level, cannot be characterised on the basis of preliminary investigation. Further

experimentation to determine the serum luteinizing hormone releasing hormone (LHRH), luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels are under way. The increase in testosterone level in the treated group indicates the increased stimulation of leyding cells, probably due to LH.

Acknowledgements

The Authors wish to thank Mr. Jamil Akhtar (Laboratory Technician) and Mr. Nazar Hussain (Laboratory Attendant) of this Institute, for their hard work during the experimentation.

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(Received: January 12, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 91-95, 1997

HEXAPROTODON IRAVATICUS FALCONER AND CAUTLEY (MAMMALIA, ARTIODACTYLA, HIPPOPOTAMIDAE) FROM UPPER SIWALIKS OF BHIMBER, AZAD KASHMIR, PAKISTAN

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Abstract: A well preserved mandibular symphysis with damaged teeth is described from Upper Siwaliks of Bhimber. It compares very favourably with the known species *Hexaprotodon iravaticus* Falconer and Cautley of the genus *Hexaprotodon*. The paper gives the detailed morphological description of the mandibular symphysis.

Key words: Siwaliks, *Hexaprotodon*.

INTRODUCTION

The *Hexaprotodon* was introduced by Falconer and Cautley (1836) as a subgenus of *Hippopotamus*. Owen (1845) elevated *Hexaprotodon* to full generic rank. According to Matthew (1929), *Hexaprotodon* was a subgenus of only doubtful validity. Colbert (1935) gave a number of arguments in support of the validity of this new genus. Uptil now two species of the genus *Hexaprotodon* are known from the Upper Siwaliks. These are *H. sivalensis* and *H. iravaticus*. According to Colbert (1935) Siwalik hippos range from Middle to Upper Siwaliks. But the recent studies of Siwalik hippos by Sarwar and Akhtar (1992) have shown that they were even present in the Lower Siwaliks. The specimen under study was collected from Upper Siwaliks during the research project sponsored by Pakistan Science Foundation.

The specimen under study has been given the number with a prefix P.U.P.C. (Punjab University Palaeontological Collection). Measurements are given in millimeters.

SYSTEMATIC ACCOUNT

Order	Artiodactyla Owen
Suborder	Suiformes Jaeckel
Infraorder	Ancodonta Matthew
Superfamily	Anthracotherioidea Gill

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Family Hippopotamidae Gray
Genus *Hexaprotodon* Falconer and Cautley

Hexaprotodon iravaticus Falconer and Cautley (Fig.1)

Type

Brit. Mus. No. 14771, a mandibular symphysis.

Horizon

"Upper Irrawaddy Beds, Lower Pleistocene", Hooijer (1950) for the type and Upper Siwaliks for the specimen under study.

Locality

"Irrawaddy River Valley, Burma", Hooijer (1950) for the type and Bhimber for the specimen under study.

Specimen under study

P.U.P.C. No. 83/748, a mandibular symphysis with damaged teeth.

Diagnosis

"The smallest of the Asiatic species, closely comparable to *Hippopotamus lemerlei* Grandidier of Madagascar as to size. Six incisors in the lower jaw; I_2 slightly above and I_3 below the level of I_1, and both not much smaller than I_1. Mandibular symphysis very long relative to its width", Hooijer (1950).

Description (Fig.1)

The type specimen is a mandibular symphysis as is the specimen under study, and the latter compares very favourably with the former. The teeth are all damaged and the symphysis is also broken posteriorly yet the typical features of the species are very evident. In the incisors only the roots are preserved which clearly indicate that I_2 is highest and I_3 is lowest in position.

This feature has also been reported by Lydekker (1884). Another feature stated by the same worker is that I_3 is laterally compressed. Hooijer (1950) contradicts this, he says, "Lydekker also emphasizes the fact that I_3 is laterally compressed, but a later specimen has disproven this view". Thus it seems to be a variable feature. In the specimen under study I_3 is almost circular in cross section. On the right side the root of the canine is preserved which is laterally compressed and vertically high. P_{1-3} are represented on the right side by their alveoli. These are elongated anteroposteriorly and each one of them is separated from the neighbouring one by a diastema. According to Lydekker (1884) :The alveoli of the three first premolars also show that pm_1 and pm_3 were separated by

Fig. 1: *Hexaprotodon iravaticus* Falconer and Cautley, a mandibular symphysis with damaged teeth (P.U.P.C.) No. 83/784), collected from Bhimber, Azad Kashmir, Pakistan (A) outer view, (B) dorsal View, (C) front view.

proportionately longer intervals". The mandibular symphysis is very long. It is thick posteriorly and thins out anteriorly.

Table: Measurements (mm) of the mandible P.U.P.C. No. 83/748.

Thickness of the mandible at the anterior end	8
Thickness of the mandible at the posterior end	69
Length of the symphysis	150
Verticle x transverse thickness of I_1	12x14
Verticle x transverse thickness of I_2	9x9
Verticle x transverse thickness of I_3	9x8
Length x width of P_1	23x15
Length x width of P_2	30x19
Length x width of P_3	31x20

DISCUSSION

The term *Hexaprotodon* was introduced by Falconer and Cautley (1936) as a subgenus of *Hippopotamus*. The main distinguishing feature of this was considered to be the number of incisors. There are six incisors in *Hexaprotodon* as compared to four in typical *Hippopotamus*. Lydekker (1884) did not accept this and placed *Hexaprotodon* as a synonym of *Hippopotamus*. Later authors Matthew (1929), Colbert (1935), Simpson (1945), Deraniyagala (1969) and Nanda (1978) contradicted this view of Lydekker (1884) and gave generic rank to the term *Hexaprotodon*. Hooijer (1950), however, did not agree with them and did not recognize *Hexaprotodon* even as a subgenus. He (1950) says, "The extinct hippopotamuses from Asia are distinguished from the living species. *H. amphibius* by a number of characters which permit of a separation of these forms from the recent species which is regarded by most modern authors as of generic value (*Hexaprotodon*). This is, of course, only a matter of taste, I prefer not to split the genus *Hippopotamus*". Not only this, he considers that in South Asia only two species existed. These are *H. iravaticus* and *H. sivalensis*. All the species except *H. iravaticus* are, according to him, the subspecies of *H. sivalensis*. These are *H. sivalensis-sivalensis, H. sivalensis-namadicus, H. sivalensis-palaeindicus, H. sivalensis duboisi* nov. subsp., *H. sivalensis-sivajavanicus, H. sivalensis-koenigswaldi, H. sivalensis-soloensis* nov. sub. sp. However, I do not agree with Hooijer (1950) and consider all of these as different and valid species.

Lydekker (1882) mentioned a mandible from Ava (Burma) under the name *Hippopotamus iravaticus* and stated that it was smaller than *H. sivalensis*. In 1883, he stated that these fossils were also found in Narbada, Sub-Himalaya. Pilgrim (1913), states, "Although the genus *Hippopotamus* is decidedly rare at the Dhok Pathan horizon, in very striking contrast to its abundance in the Tatrot beds, yet there seems, to be undoubted evidence that it does occur. The species *Hippopotamus iravaticus* occurs in Burma, where it is said to be associated with fossils of a Middle Siwalik type at the very base of the Irawady series and the same species seems to have been found in the Hasnot beds, while teeth of the same genus come from Dhok Pathan itself". The specimen under

study is from Bhimber - Upper Siwaliks. Pilgrim (1910) mentioned *Hippopotamus iravaticus* occurring in Yenangyaung, Burma which he stated to be of Hasnot horizon. Colbert (1935) considers, "Irrawaddy Beds, equivalent to the Middle Siwaliks". Thus, it seems that this species occurs in Middle and Upper Siwaliks. Tatrot zone, according to Colbert (1935) is lower part of Upper Siwaliks.

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(Received: February 20, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 97-106, 1997

EFFECT OF DICHLORVOS ON THE BRAIN AND FAT BODY CELLS OF THE ADULT *MUSCA DOMESTICA* L. (MUSCIDAE : DIPTERA)

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Abstract: LD_{50} for dichlorvos was calculated to be 11.8 ppm after 24 hours of treatment. Of all the components of the brain the neurons were the most affected. They showed hypertrophy and pyknosis which ultimately led to the histolysis of the cells. Glial cells, although, not so severely affected, changed in their nuclear contents to some extent. Neural lamella thickened irregularly after treatment. The neuropile along with its glomerular bodies underwent vacuolization. The plasma membranes of the fat body cells ruptured after extensive vacuolization and the cytoplasmic content almost disappeared. The chromatin material darkened and became concentrated in the middle of the cells.

Key words: Insecticide toxicity, organophosphate, housefly, LD_{50}, cell morphology.

INTRODUCTION

Musca domestica (housefly) is a cosmopolitan non-biting muscid fly. Its abundance varies seasonally throughout the year. It oviposits on a variety of decomposing materials, such as fruits, vegetables, animal viscera, marine animals etc (D'Almedia and Mayio, 1993). Usually some 500-1000 eggs are deposited by a female during its lifetime, while its life cycle may be completed within 11 to 49 days depending upon the temperature. It can transmit a large number of diseases to man. Over 100 species of pathogens have been recorded as being carried by it (Service, 1980), some of them such as diarrhoea can be fatal especially for children.

In order to eliminate this pest, various methods can be undertaken; physical and mechanical control, environmental sanitation, biological and chemical control. The chemical control is the most popular method ranging from chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids.

Generally, pesticides have a specific mode of action and toxicity (Corbett, 1974; Matsumura, 1975). The organophosphates can be categorized as stomach and systemic poisons, some also as abrasive compounds and contact piosons, while others as fumigants (Hassal, 1983). They primarily act upon the nervous system. An important feature of this group is that different members possess very different physicochemical

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properties, in particular, they have different vapour pressure at room temperature and different stabilities in water. They also vary considerably in the chemical stability and their toxicity to mammals (Hassall, 1983).

Dichlorvos which was used in the present study is an organophosphate compound. It is regarded as a low persistance, contact poison with a vapour pressure sufficiently high to enable it to act in the vapour phase, *e.g.* for protection of stored products. It decomposes rapidly in animals, and consequently has less harmful effects (Hassall, 1983).

present study its effect on the different components of the brain of *Musca domes* as been studied.

MATERIALS AND METHODS

Musca domestica, used for the present research work, were collected and reared as described previously (Sharif and Ali, 1996). The newly emerged flies were kept separately and fed on a diet of milk-sugar solution. This was to maintain the uniformity of the flies to be used for experimental purposes.

Insecticide and its treatment

Dichlorvos which is sold under the trade name of Nogos was obtained in commercial formulation of 50EC (Emulsifiable concentration) from Ciba Geigy. It is a contact and stomach poison with fumigant action, and is used specifically for the control of houseflies (Hassal, 1993). The insecticide was diluted five time in distilled water and was used as the stock solution. This was further diluted to get 5 ppm and 10 ppm solution. The adult flies were kept in batches of 10 each in glass jars. Insecticidal dose was given to the insects by pouring 1 ml of it over the sugar granules given as food. About 1 g of sugar was used for each batch. Control insects were given equal amount of distilled water mixed in 1 g of sugar. The flies were taken out at intervals of 1 hour (10 ppm) and 2 hour (5 ppm) after dichlorvos treatment for histological preparations. LD_{50} was calculated by probit analysis (Finney, 1952; Busvine, 1971).

Histological studies

The head was fixed in Bouin's fixative, dehydrated in ascending grades of alcohol, cleared in cedar wood oil and embedded in paraffin wax. Serial sections 8-10 μm thick were cut with a rotary microtome. Sections were stained in hematoxyline and counter stained with eosin.

RESULTS

The toxic effect of dichlorvos (5 ppm and 10 ppm) on the histology of the brain was studied after various time intervals. The earlier signs of the syndrome appeared

shortly after the treatment and the intensity increased with prolongation.

Cortex

The general sensory neurons and the few motor neurons found in the brain show hypertrophy and their chromatin contents start clumping thus forming irregular lumps near the periphery of the nuclei. The effects of treatment with 5 ppm after 6 hours and with 10 ppm after 3 hours respectively (Tables I and II), are almost the same but the brains of the insects treated with the higher dose show extensive cellular histolysis and ultimate disintegration.

Motor neurons

These cells show some increase in the size of nuclei after treatment. However, the nuclear contents undergo the most conspicuous changes, starting with the clumping of the chromatin material and irregular lump formation around the inner side of the nuclear membrane. Treatment with 5 ppm after 6 hours and 10 ppm after 3 hours results in intense histolysis and ultimately disintegration of the cellular contents.

Sensory neurons

Optic ganglion neurons

These cells show significant increase in size. Their cell boundaries become distinct and nuclei start undergoing pyknosis after 3 and 6 hours when treated with 10 ppm and 5 ppm of the insecticide respectively. The chromatin forms clumps in most of the optic neurons.

Globuli cells of corpora pedunculata

The globuli cells also show considerable hypertrophy and necrosis. The cell boundaries of these neurones also became very clearly defined after 2 and 4 hours of treatment with 10 and 5 ppm respectively. the chromatin material becomes scattered which later on becomes scarce, the nucleoli also became more conspicuous.

General sensory cells

These cells also become considerably hypertrophied with significant increase in the size of the nuclei which is accompanied by necrosis. However, unlike motor neurons none of the sensory type cells undergo complete histolysis and disintegration. The contents of the nuclei show similar charges as that of the other neurons.

Table I: **Dimensions of nuclei of different brain cells in control of *Musca domestica* treated with 5 ppm dichlorvos for 6 hours (each reading is a mean of 30 values)**

Cell types	Control		Dichlorvos treatment (hours)					
			2		4		6	
	L	W	L	W	L	W	L	W
Glial Type I	5.50[a] ±0.68	3.00 ±0.71	6.30 ±0.87	3.00 ±0.55	7.00 ±0.55	3.00 ±0.55	7.00 ±1.11	3.00 ±1.04
Glial Type II	3.00 ±0.88	3.00 ±0.68	3.00 ±0.68	3.00 ±0.32	5.00 ±0.88	4.00 ±0.68	4.00 ±0.96	3.00 ±0.31
Glial Type III	15.50 ±2.73	10.00 ±0.88	19.50 ±4.80	14.00 ±3.30	15.50 ±2.73	11.00 ±2.65	14.00 ±2.36	11.00 ±1.44
Glial Type IV	5.00 ±0.55	2.00 ±0.27	5.00 ±1.04	4.00 ±0.17	5.00 ±0.63	2.50 ±0.00	6.00 ±1.44	3.00 ±0.28
Motor neurons	10.50 ±3.60	8.00 ±1.60	12.00 ±1.85	9.50 ±2.09	11.00 ±2.85	9.50 ±2.09	12.00 ±1.89	9.90 ±1.53
Optic ganglion neuron	2.50 ±0.55	2.00 ±0.31	3.00 ±0.22	3.00 ±0.27	2.50 ±1.11	3.00 ±0.22	4.50 ±0.75	4.50 ±0.74
Globuli cells	3.00 ±0.36	3.00 ±0.55	4.00 ±0.29	3.00 ±0.62	5.00 ±0.35	3.50 ±0.41	6.00 ±0.88	5.00 ±0.55
General sensory cells	3.00 ±0.95	3.00 ±0.32	3.00 ±0.45	3.00 ±0.29	6.00 ±0.94	5.50 ±0.72	7.00 ±1.22	5.00 ±0.55
Neurosecretory cells	10.00 ±3.11	6.00 ±1.25	11.00 ±3.22	8.00 ±2.39	10.00 ±2.23	9.00 ±1.36	11.00 ±1.44	8.00 ±1.44
Fat body cell nuclei	8.50 ±2.85	7.60 ±2.50	11.00 ±1.25	9.00 ±1.44	11.50 ±1.36	10.00 ±1.76	16.00 ±3.75	14.50 ±3.60

[a]Mean ± SEM

Abbreviations used: L, length; W, weight.

Table II: **Dimensions of the nuclei of different brain cells in control of *Musca domestica* treated with 10 ppm dichlorvos for 3 hours (each value is a mean of 30)**

Cell types	Control		Dichlorvos treatment (hours)					
			1		2		3	
	L	W	L	W	L	W	L	W
Glial Type I	5.05[a] ±0.68	3.00 ±0.71	5.00 ±0.55	3.30 ±0.68	7.00 ±1.67	3.50 ±1.85	6.00 ±0.88	2.00 ±0.68
Glial Type II	3.00 ±0.88	3.00 ±0.68	7.00 ±0.72	6.00 ±0.72	6.00 ±0.88	5.50 ±0.68	8.00 ±1.42	6.50 ±2.23
Glial Type III	15.50 ±2.73	10.00 ±0.88	20.50 ±4.10	10.00 ±2.88	18.00 ±4.80	14.00 ±3.95	23.00 ±5.70	17.00 ±6.47
Glial Type IV	5.00 ±0.55	2.00 ±0.27	5.50 ±0.35	2.50 ±0.22	5.50 ±1.11	3.00 ±0.68	6.00 ±1.11	2.00 ±0.55
Motor neurons	10.50 ±3.60	8.00 ±1.60	9.00 ±2.09	8.00 ±1.21	9.00 ±0.88	8.00 ±0.55	10.00 ±1.76	7.00 ±1.62
Optic ganglion neuron	2.50 ±0.55	2.00 ±0.31	2.50 ±0.22	2.50 ±0.00	3.00 ±0.86	3.00 ±0.68	4.00 ±1.46	3.00 ±1.03
Globuli cells	3.00 ±0.36	3.00 ±0.55	5.57 ±0.68	5.00 ±0.87	5.50 ±0.68	4.50 ±0.78	6.00 ±1.04	5.50 ±1.21
General sensory cells	3.00 ±0.95	3.00 ±0.32	4.00 ±0.80	3.50 ±1.20	5.50 ±1.17	5.00 ±1.54	7.00 ±1.85	5.00 ±1.67
Neurosecretory cells	10.00 ±3.11	6.00 ±1.25	11.00 ±1.62	10.00 ±0.55	8.00 ±1.11	8.00 ±1.11	12.00 ±2.59	8.50 ±1.36
Fat body cell nuclei	8.50 ±2.85	7.60 ±2.50	14.00 ±2.36	9.00 ±1.44	13.50 ±1.36	12.00 ±1.86	13.00 ±3.11	11.50 ±2.05

[a]Mean ± SEM

Abbreviations used: L, length; W, weight.

Neurosecretory cells

The effect of intoxication on these cells is more like the motor neurons. General hypertrophy, scattering of the chromatin material, which is centrally placed in the control insects, and extensive vacuolization of the cytoplasm happens soon after treatment. All ends in more or less complete histolysis.

Glial cells

The glial type I cells also show slight increase in size while the neural lamella along which these cells are located becomes thickened unevenly. The few glial type II cells identified in the present study did not show any significant change in the size. The nuclei of the type III glial cells also show no significant increase in the size. However, their chromatin material became somewhat scattered. Apart from some scattering of the chromatin material glial type IV cells also do not undergo any significant change.

Neuropile

Upto two hours of the treatment no perceptible change in the neuropile was detected. But after 2 and 4 hours of treatment with 10 ppm and 5 ppm respectively vacuolization started which gradually intensified and became considerable towards the end of the experimental period.

Fig.1: **T.S. of brain of control *Musca domestica*, showing neurosecretory cells and some other features in the region of pars intercerebralis. a, axons; NC, neurosecretory cell; nl, neural lamella; N, neuropile. 40 x.**

Fig.2: **T.S. of brain of *Musca domestica*, showing motor neurones with scattered chromatin material (5 ppm dichlorvos, 2 hours after treatment). n, nucleus; cyt, cytoplasm; mn, motor neurones. 100 x.**

Fig.3: **T.S. of brain of *Musca domestica*, showing neurosecretory cell and pyknotic neurones (5 ppm dichlorvos, 4 hours after treatment). cyt, cytoplasm; n, nucleus; NS, neurosecretory cell; pn, pyknotic neurones.**

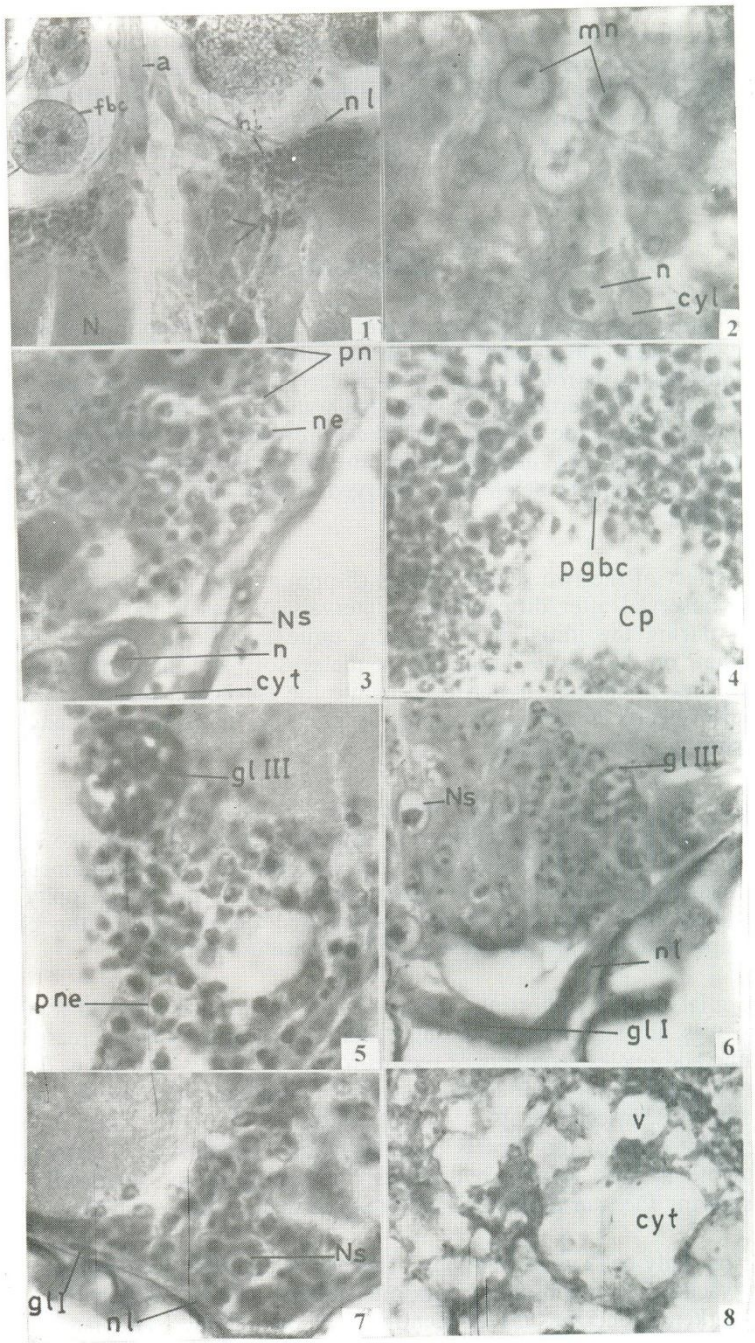
Fig.4: **Pyknotic globuli cells of experimental brain (5 ppm dichlorvos, 4 hours after treatment). cp, corpora pedunculata; gbc, globuli cells. 100 x.**

Fig.5: **Glial type III cell with clumped chromatin material (5 ppm dichlorvos, 4 hours after treatment). gl III, glial type III cells; pgsc, pyknotic general sensory cells. 100 x.**

Fig.6: **T.S. of experimental brain showing neurosecretory cells with dispersed chromatin material. Thickened perilema and glial type I cells can also be seen. (5 ppm dichlorvos, 4 hours after treatment).**

Fig.7: **T.S. of ventral portion of protocaebrum showing neurosecretory cells. Glial type II cells, Glial type I cells and reural lamella are also seen (5 ppm dichlorvos, 4 hours after treatment). NS, neurosecretory cells; gl I, glial type I cells; nl, neural lamella. 100 x.**

Fig.8: **Fat body cells of experimental insect showing vacuolated cytoplasm and ruptured cell membrane (5 ppm dichlorvos, 6 hours after treatment). cyt, cytoplasm; n, nucleus; v, vacuole. 100 x.**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Fat body cells

The fat body cells are the most pronouncely effected by the insecticide. The immediate effect was on the cytoplasm which became vacuolized at the very beginning of the treatment. Treatment with both the doses resulted in vacuolization and ruptured cell walls. The chromatin material became scarce and these cells which are multinucleate in the normal insects showed only a small mass of chromatin material thus giving the appearance of being uninucleate.

DISCUSSION

The common housefly (*Musca domestica*) has a cosmopolitan distribution and is important as a mechanical carrier of various infectious agents (Lapage, 1962; Service, 1980). Considering its role as a mechanical carrier of different diseases various control measures are being undertaken to control the houseflies. In the present study, dichlorvos was used as an insecticide to study the effects it caused on its brain and fat body. During the determination of LD_{50} after 24 hours, the normal syndrome of neural poisoning like tremors and the ultimate death was observed as also reported by earlier workers like O'Brien (1960).

The effect of the two selected doses (5 ppm and 10 ppm) on the brain and the fat body was observed after different time intervals. It was found that the results of the poisoning during the initial 6 hours were quite significant. The histological studies revealed that, on the whole, the nuclei of the sensory and motor neurons were effected the most. They underwent hypertrophy while the chromatin material became clumped. As the sensory cells are the most commonly present cells in the brain any adverse effect on them showed up very clearly.

The neuropile also showed considerable vacuolization, which indicated that the synapses and different nerve tracts were affected. Other workers like Ali and Ahmad (1982) using different insecticides have also reported the same phenomenon for various insects. According to Teleferd and Mastsomura (1970), the hypertrophy is due to the fact that the cell membranes became adversely effected, hence the changes in the membrane permeability are the cause of the increase in size.

The glial or the supporting cells were not very much affected as the increase in their size was not significant, although their nuclei somewhat increased in size. The cytoplasmic and nuclear contents of the neurosecretory cells also showed adverse effects indicating disturbance in their secretory activity, although their size did not significantly change. As this insecticide is a neural inhibitor, its mild form of action on the neurosecretory cells of the flies is understandable. Dichlorvos is particularly effective against cholinesterase which hydrolyses the acetylcholin generated in myoneural junctions during the transmission of motor commands (Hartley and West, 1969).

The fat body cells were generally affected. Their plasma membranes ruptured after extensive vacuolization and the cytoplasmic contents almost disappeared. The chromatin material darkened and became concentrated in the middle. The fat body which is a

general food reservoir (Richards and Davies, 1977) was thus badly damaged.

The present results suggest that, dichlorvos is an effective neurotoxin. It starts its action very quickly and leaves considerable effects on the brain tissue within hours, unlike dieldrin which damaged the nervous tissue of the cockroach after 24 hours of treatment with a much higher dose (Ali and Ahmad, 1982). The development of resistance to the various insecticides in insects when their cholinesterase activity rises significantly is a very common phenomenon as shown by various workers like Hirashima *et al.* (1989); Shakoori and Saleem (1991) and Mourya *et al.* (1993).

However, in the present studies this insecticide was quite effective, so it seems that the flies have not yet developed resistance against it.

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(Received: May 26, 1997)

Punjab Univ. J. Zool. Vol. 12, pp. 107-113, 1997

PREDOMINANCE OF LEAD RESISTANT AND LEAD PROCESSING BACTERIA IN INDUSTRIAL AND SEWAGE WASTES

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Abstract: Twenty six samples from industrial wastes and sewage waste were collected and checked for the presence of lead resistance bacteria. Nineteen lead resistant bacterial strains were isolated. Most of the bacteria showed resistance against lead up to a concentration of 1 mg/mL of the medium. Seven bacterial strains were selected to determine their lead processing ability. The bacteria showed efficient capability to process lead. The mechanism for lead processing is proposed to be lead accumulation or lead sorption. Variation in the lead processing ability of the bacteria was observed. Most of the isolates showed an initial speedy processing of lead during 24 hours and a slower relatively constant processing during next 48 hours. One of the isolates showed a slow processing during first 24 hours and a more enhanced processing during the next 24 hours and a very speedy processing during the next 24 hours. Plasmids were detected in some of the strains capable of lead processing showing that the gene for lead processing was usually present on chromosomal DNA. Plasmid occurrence frequency was more in an area where there was constant inpouring of industrial wastes for the last many (~50) years while it was less in other areas where there were separate ponds.

Key words: Biodegradation, heavy metals, lead resistance, lead accumulation, industrial pollution.

INTRODUCTION

Lead resistant bacteria are frequently found in sewage water and industrial wastes containing lead. A number of scientists have reported isolation of lead resistant bacteria (Couillard and Chartier, 1993; Tondwalkar *et al.*, 1990; Bender *et al.*, 1989; Lee *at al.*, 1992). Most of the developing countries like Pakistan are facing the problem of heavy metal contamination through industrial wastes while these wastes are not being treated properly. With increasing insight into the capability of a variety of microorganisms to process and detoxify heavy metal ions, the scientists have suggested to exploit this capability in environmental clean up operations. Bioremediation or the use of microorganisms for decontamination is getting the status of first rate method for environmental protection. Microorganisms used for these

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operations not only detoxify heavy metal ion in the environment but also don't add any surplus chemicals as is usually the result of chemical treatment plants. Metal processing strains have now been commonly reported in a number of studies. These strains have been studied and improved for exploitation in detoxification procedures. Lead resistant bacteria have also been used for lead and other metal ion processing. In a study bacterial strains were used to adsorb metals like lead, copper and zinc from spent wash. One of the strains removed 64% lead in one hour and 82% copper in two hours, while the other strain removed 76% of the zinc in two hours (Tondwalkar *et al.*, 1990). Another study reports the use of a technique of integrated ecosystem for the uptake of lead. The transfer of metal through the system was observed with the eventual binding of the metal with the biomass. It was noted that increase in biomass of microorganisms increased lead-recovery. It also increased microbial tolerance for lead. The stable microbial mass floating on the surface of the pond bound the metal for extended period of time (Bender *et al.*, 1989).

The aim of this study was to check the presence of lead resistant and lead processing bacteria in various sewage wastes and industrial effluents. In this regard nineteen bacterial isolates from water samples got from various industrial waste were checked for their lead resistance. Seventeen bacterial isolates showed resistance against high concentrations of lead in the medium. Lead processing ability of the isolates was checked by estimating the concentration of lead in the medium after various intervals of growth of bacteria in the cultures. The reduction in the amount of lead indicated the processing of lead by bacterial strains. Two modes of processing of lead were observed. The implications of the lead resistant bacteria in bioremediation and environmental cleanup operations were discussed.

MATERIALS AND METHODS

Collection of samples

Twelve water samples were collected from effluents released by tanning industry, 4 from effluents of industry engaged in food processing, soap formation and textile mixed with sewage waste and three from effluents of ICI paint plant. The samples were collected in sterile screw capped glass bottles, brought to the laboratory and stored at room temperature before spreading on plates.

Growth and lead resistance of bacteria

For selection of lead resistance bacteria LB agar plates with 1mg/mL lead were used. Lead acetate was used as source of Pb^{2+}. Lead acetate solution and LB agar medium were autoclaved separately and allowed to cool down. When the temperature of the two solutions was slightly less than 60°C, the solutions were mixed and poured into

plates. Industrial effluent (100µl) was spread on the plates and the growth of colonies was observed after 24 hours. Colonies were picked and streaked for purification and for determination of maximum resistance of the strains against lead.

Estimation of lead in the medium

In order to estimate the amount of lead in the medium, dithizone method (E. Merck) was used. Reagent I (R1) was prepared by dissolving 10mL of hydrazinium hydroxide in 70mL of 1N HCl, then adding 20g of NaCl and making the volume up to 100mL with distilled water. Reagent 2 (R2) was prepared by dissolving 20g potassium hydrogen carbonate, 5g potassium cyanide, 5g potassium sodium tartarate and 25mL ammonium solution in 100mL of distilled water. Dithizone solution was prepared by dissolving 15mg of dithizone in 1000mL of chloroform. Five mL of LB broth with lead concentration of 1mg/mL was taken in test tubes. The tubes were inoculated with fresh bacterial cultures. After incubation for 24 hours 1mL culture was taken from the tube aseptically. The culture was diluted to 25 times volume with distilled water. This mixture was taken in a separating funnel and 2.5mL of R1 were added followed by addition of 2.5mL of R2. After that 12.5mL of dithizone solution was added. The mixture was shaken for 5 minutes and the pressure developed by shaking and reaction was released by removing the stopper or valve. The mixture was allowed to stand for a few minutes. Two layers were formed. The lower layer containing chloroform was collected in a glass bottle and OD of the solution was taken at 515nm against a blank which was prepared through a similar procedure by taking 25mL distilled water without any culture. The same procedure was adopted to estimate lead at 0, 48, and 72 hours. Control of the lead processing was run using the medium without inoculation incubated at same conditions as that of the culture.

Amount of lead was calculated by the following formula:

$$G = \frac{M \times 95.4 \times \text{dilution factor}}{a}$$

where G = amount of lead in mg/L, a = amount of water used in mL, M = absorbance. Bacterial processing of lead was assessed by estimating the amount of lead in the medium after various time intervals. All the readings were taken in triplicate for statistical analysis.

Isolation of plasmids

A single colony of various lead resistant strains was selected and grown in LB liquid medium for plasmid isolation. The procedure adopted for isolation of plasmids was as described by Holmes (1984). Plasmid DNA isolated from the bacterial isolates

was run on agarose gel and visualized under UV illuminator after staining with ethidium bromide. The isolation experiment was repeated three times to ascertain the presence of the plasmids.

RESULTS

Isolation of lead resistant bacteria

LB agar plates containing lead at a concentration of 1mg/mL were used to isolate lead resistant bacterial isolates. Seventeen isolates showed resistance against lead concentration of 1mg/mL while two isolates showed resistance against lead concentration of 2mg/mL of the medium. Single colonies were picked and inoculated in 250mL flasks containing 100 mL of the LB liquid medium. The pH of the medium was adjusted at 7.0 and the temperature of incubation was 30°C.

Processing of lead by bacterial isolates

Processing of lead by the bacterial strains was determined though dithizone method. Five strains were used for checking lead processing efficiency. The results are shown in Table I and Fig. 1.

Table I: Percentage reduction after various time intervals in amount of lead present in the medium inoculated with fresh cultures of bacterial isolates

	Time (Hours)		
Isolate No.	24	48	72
	Lead Reduction (%)		
	66.33*	67.43	68.43
2	64.23	64.79	65.12
3	69.53	76.37	76.93
4	2.64	30.0	79.0
7	50.0	52.64	55.29

*All the values in the table are mean of three readings

Isolation of plasmids

Twelve isolates from the effluents of Kasur tanneries waste were used for plasmid isolation and nine (75%) out of these were found to harbor plasmids. Three isolates from ICI paint plant wastes were used for plasmid isolation and none of these was found to harbor a plasmid. Four isolates from effluents of Kot Lakhpat industrial area of Lahore were used for isolation of plasmids and only one strain (25%) was found to contain a plasmid.

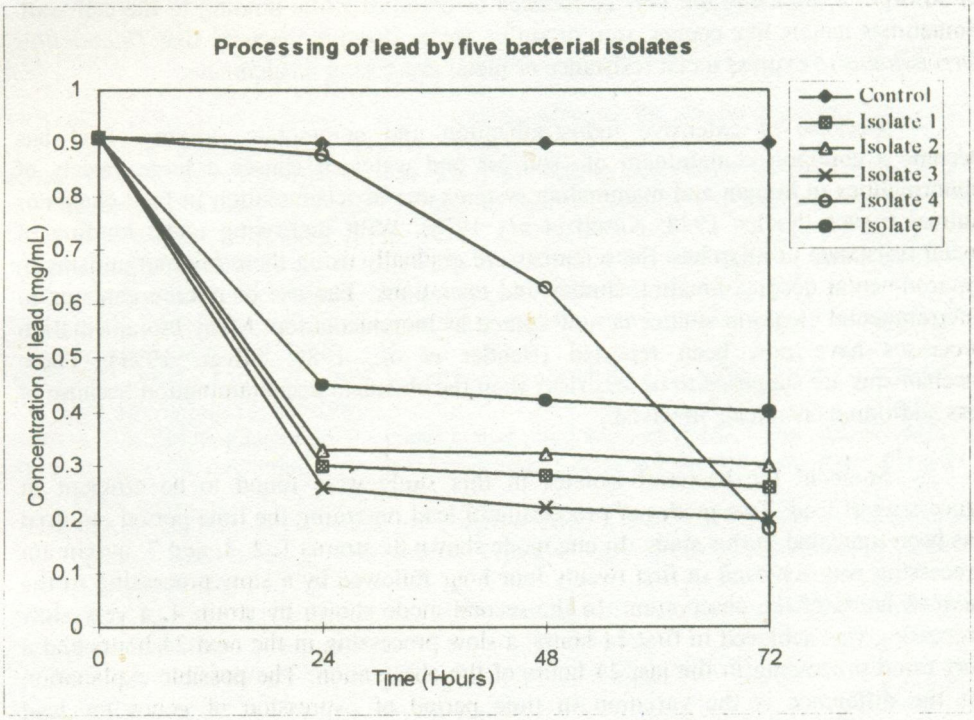

Fig. 1: Graph showing the amount of lead in the media estimated at various time intervals after inoculation of the media with exponential phase cultures of various bacterial isolates.

DISCUSSION

Metal tolerant microbial strains have been frequently reported in water samples taken from the environment receiving metal ions. They originate in habitats having elevated levels of heavy metals (Verma, 1995). Bacterial resistance mechanisms have

extensively been studied and it is found that bacteria resist metals due to the presence of some cellular mechanisms of combating toxic effects of metals (Mergeay, 1991; Nies and Silver, 1995; Wood and Wang, 1985). Various metal resistance and metal processing mechanisms are involved in bacterial metal resistance. Generally these are reduction of metal and detoxification through the enzymes present at the cell wall or periplasmic space. In this process the reduced metal ions remain outside the cell membrane and usually in the medium. Another method of metal resistance is metal accumulation by the bacterial cells through ion influx and effluxes. In this process the metal ions sometimes accumulate inside the cell and may cause ultimate death of the cell. Still other method is biosorption or binding at the surface of the cell. In this process of adsorption the metal ion may be reduced or oxidized while binding to the cell wall. Sometimes metals like copper, iron or sulfur are oxidized by bacteria like *Thiobacillus ferrooxidans* to express metal resistance or metal processing mechanisms.

Because of extensive industrialization and automobile running, lead has become a common contaminant of soil air and water. It causes a large variety of abnormalities in human and mammalian systems due to accumulation in food chains or animal tissues (Sachs, 1978; Kusell *et al.*, 1978). With increasing understanding of metal resistance in microbes, the scientists are gradually using these microorganisms in environmental decontamination studies and operations. The use of microorganisms in environmental clean-up studies is now coined as bioremediation. Many bioremediation processes have now been reported (Bender *et al.*, 1989; Silver, 1994). These mechanisms are supposed to be less risky than the chemical decontamination because of less additional chemicals involved.

Some of the bacterial isolates in this study were found to be efficient in processing of lead. Two modes of processing of lead regarding the time period required has been indicated in this study. In one mode shown by strains 1, 2, 3, and 7, maximum processing was achieved in first twenty four hour followed by a slow processing in the next 48 hours of the observation. In the second mode shown by strain 4, a very slow processing was achieved in first 24 hours, a slow processing in the next 24 hours and a very rapid processing in the last 24 hours of the observation. The possible explanation for the difference is the variation in time period of expression of genes for lead processing. Secondly, some of the bacteria exposed to constant stress of lead may have developed an efficient system of lead processing which comes in operation quickly when exposed to lead while others may have to undergo a long genetic process for expression of lead processing proteins. Another conclusion which can be drawn from the results is that resistance to lead and processing of lead may be achieved through two different mechanisms.

Most of the strains got from tannery effluents were found to harbor plasmids which indicate that plasmid transfer took place in an area due to common environment

and contact of bacterial strains with one another. Secondly, the absence of plasmids in lead resistant strains also indicated that gene for lead resistance is not solely present on plasmids. It is present on the chromosome. The absence of plasmids in bacterial isolates from other effluents like that of ICI showed that the bacteria in the area had less possibility of encounter with plasmid bearing bacteria or the proper stress was not present for maintenance of plasmids.

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(Received: January 15, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 115-125, 1997

EFFECTS OF ANDROGENS ON NUCLEIC ACIDS AND PROTEIN CONTENTS IN REGENERATING EXTENSOR DIGITORUM LONGUS MUSCLE FOLLOWING ORTHOTOPIC TRANSPLANTATION IN RATS*

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Abstract: Effects of testosterone, on regeneration of muscle fibres within rat Extensor digitorum longus (EDL) muscle grafts were studied under various experimental conditions. It was discovered that the muscle grafts reacted negatively to lack of androgens. The RNA and total protein contents of the grafts in the gonadectomized rats were less than those found in controls. EDL muscle grafts in testosterone propionate replaced rats developed much better than those in the androgen deprived ones. These results suggest that mammalian skeletal muscle is, in general, sensitive to the presence or absence of androgens while regenerating following free transplantation.

Key words: Gonadectomized, testosterone propionate, muscle grafting.

INTRODUCTION

Free skeletal muscle transplantation has, extensively, been studied in various animals (Mufti *et al.*, 1977; Grim *et al.*, 1986; Roberts *et al.*, 1989; Qazi and Mufti, 1990) and used therapeutically in a number of cases involving different muscles (Hakelius, 1974a,b; Grotting *et al.*, 1990; Chuang *et al.*, 1994). Process of skeletal muscle fibre regeneration following the grafting, in general, enables the graft to achieve about 50% of its original structural and functional characteristics (Wagner *et al.*, 1977; Faulkner and Côte, 1986; Ontell, 1986). Various parameters have been elaborated to determine the success of a free muscle transplant, viz mass, number and diameter of regenerated muscle fibres, enzyme profiles and physiological characteristics (Faulkner *et al.*, 1981; Côte and Faulkner, 1984; Faulkner and Côte, 1986; Ontell, 1986; Carlson, 1988).

Among these measures, DNA, RNA and total protein contents of regenerating skeletal muscles have received practically little attention and only a few studies can be enlisted in this regard (Jones, 1983; Friedman *et al.*, 1986). However, recently Gill and

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0079-8045/97/0001-0113 $ 03.00/0

*Part of Ph.D. thesis of first author, submitted to the University of the Punjab, Lahore, Pakistan

Shakoori (1995) have reported that these biochemical parameters are more important determinants of the success of a free muscle graft.

Owing to the fact that a freely transplanted muscle regenerate remains deficient in its various characteristics, efforts have been made to improve quality of the regenerate through different postgrafting interventions (White *et al.*, 1981; Mufti and McNemar, 1986; Weiss and Oron, 1992; Bischoff and Heintz, 1994). In this connection Qazi and Mufti (1989, 1990) have initiated studies pertaining to the influence of some anabolic hormones on regeneration of skeletal muscle fibres following the grafting.

Effects of anabolic hormones on growth and maturation of skeletal muscles are well known (Gustafson *et al.*, 1986; Bates and Holder, 1988; Boissonneault *et al.*, 1989; Balon *et al.*, 1990). And among these hormones testosterone has been most thoroughly studied and discovered to be highly myotropic (Kumar *et al.*, 1981; Kuhn and Max, 1985). Griffin and Wilson (1985) have described that a major component of androgen-induced weight gain and nitrogen retention in hypogonadal man is an increase in skeletal and muscle mass. Application of this hormone results in an increase in the protein synthesis as well as a decrease in the protein degradation within the skeletal muscle (Goldberg *et al.*, 1975; Crist *et al.*, 1983). Testosterone and its derivatives have been applied clinically for a number of years as general anti-catabolic agents to counter negative nitrogen balance *e.g.*, during muscle wastage (Rothstein and Rose, 1982). Levator Ani (LA) has long been considered an index of the myotrophic activity of androgenic and/or anabolic hormones (Eisenberg and Gordon, 1950). A greater concentration of androgen receptors in the LA than other skeletal muscles (Rance and Max, 1984) could contribute to the importance of the androgenic response of the muscle. Rand and Breedlove (1992) have found that androgen exerts its anabolic effect by acting locally upon a cell population within or near the bulbocavernosus and levator ani muscles. LA has also been shown to retain its androgen sensitivity during its regeneration (Carlson *et al.*, 1979; Max *et al.*, 1981; Mufti, 1985).

Apart from effects of androgens on skeletal muscles, in general, and on regenerating LA muscle, there is little information, about their role on regenerating muscles which are not known specifically sensitive to these substances. The present work was, therefore, intended to study the effects of androgens on regenerating extensor digitorum longus muscle and this paper reports response of the muscle transplants in terms of DNA, RNA and total protein contents in gonadectomized and testosterone propionate replaced rats. This information is helpful in improving the regeneration of free skeletal muscle grafts in hypogonadal patients.

MATERIALS AND METHODS

Animals

Adult male rats (*Rattus norvegicus*, Sprague-Dawley strain), weighing 100-300 g were obtained from National Institute of Health (Islamabad) and kept in standard animal room facilities with roughly 12 hours dark/light cycle. They were fed commercially prepared food and given a constant supply of drinking water. The food contained 1)

poultry feed, 5 kg; 2) fish meal, 1 kg; 3) wheat flour, 2 kg; 4) molasses, 100 g; 5) water, 3 lit.

Surgical procedure

All surgeries were performed under disinfected conditions. The rats were anaesthetized with ether both at the time of transplantation and while recovering the grafts. The operated animals were supplied with 0.06% terramycin in their drinking water for 3-4 days postoperative. The operated animals were kept in cages and fed routinely.

Gonadectomy

Male rats were gonadectomized 4-days prior to the muscle transplantation, so as to eliminate residual testosterone within the blood. In this process the ventral aspect of scrotum of the anaesthetized animals was first shaven clean and wiped with 70% alcohol. A median incision was given along the raphe seen on ventral side of the scrotum. The internal fascia were then cut through to reach the testes which were then pulled out of tunica vaginalis, along with whole of epididymis and part of vas deferens. Since all this area is highly vascularized, a tight knot was applied proximal to the level at which the epididymis and vas deferens were severed, so as to avoid excessive bleeding during removal of the testis. Tunica vaginalis was sutured close and then various fascial sheaths were sutured with 6-0 silk and finally the two cut ends of the skin were sutured with 4-0 silk. The sutured area was wiped again with 70% alcohol.

Transplantation of extensor digitorum longus (EDL) muscle

This muscle was orthotopically transplanted, both in control and experimental animals. All experimental rats were castrated as described above. The EDL muscle was isolated from its surrounding associations such as neural and vascular connections and its proximal and distal tendons were cut leaving a small tendinous stump behind. The muscle was taken out, weighed and then grafted back in its original bed in proper orientation. Both proximal and distal tendons were sutured with respective stumps with a 6-O silk. This was followed by suturing the fascia, again by 6-O silk and finally the two cut ends of the skin were carefully and closely sutured with 4-O silk.

At each prescribed interval, ranging from 1 to 4 weeks postgrafting, the grafts were isolated, removed, weighed and processed for the biochemical analyses.

Effects of androgens

The EDL muscle grafts in gonadectomized (G) rats were compared with those of uncastrated control rats (C). Testosterone propionate anhydrous (Sigma) was dissolved in such a way that 0.1 ml of corn oil contained 0.1 and 1.0 mg of it. The drug was administered intra-peritoneally, each day starting from the day of muscle transplantation in orchidectomized rats. Following orthotopic transplantation of both EDL muscles, two groups of the rats received 0.2 mg/100 g body wt. (GTP-I) and 2.0 mg/100 g body wt.

(GTP-II) doses of testosterone propionate. EDL muscle grafts in vehicle injected gonadectomized rats (GVC) served controls for this series of experiments.

Biochemical analyses

Total protein contents of the EDL muscle were assessed by the methods of Lowry *et al.* (1951) as modified by Miller (1959) and Schacterle and Pollack (1973). DNA and RNA contents were estimated by methods of Wannemacher *et al.* (1965), Shibko *et al.* (1967) and Munro and Fleck (1969) as modified by Lone and Matty (1980). Purified calf thymus DNA, calf liver RNA and bovine serum albumin were used to prepare standard curves.

Statistical analyses

The mean values of experimental EDL muscle grafts were compared to respective controls by applying Student's "t" test and single-factor analysis of variance. Following the analysis of variance the detailed comparisons were made according to Campbell (1989). Standard curves were drawn after applying regression equations.

RESULTS

Weight of animals and EDL muscle grafts

No change was observed in pattern of body weight gain between control and gonadectomized rats and at end of the experiment the animals showed 17.45 and 17.97% gain in body weights, respectively. Concerning the weight of EDL muscle grafts a significant decrease in the gonadectomized rats was found in 3 week old grafts only. Percent recovery in weights of the regenerates (mg/100 g body wt.) at this stage was turned out as 48.21 ± 2.17 and 32.24 ± 4.72 for the control and gonadectomized rats, respectively.

In case of testosterone propionate replaced rats there had been no significant difference in % gain in body weights from that of GVC rats except at 3 week postgraft period where 1.17% weight loss was observed for GTP-II animals, while the GVC rats attain 8.13% gain in their weights. At 2-week stage % recovery in weights of the muscle regenerates was found as 40.67 ± 3.45, 44.38 ± 3.36 and 57.28 ± 2.77 for GTP-I, GTP-II and GVC rats, respectively, and the values for the testosterone propionate replaced series of rats are significantly less than that of GVC rats. However, values of the percent recoveries of 3-week GTP-I and 4-week GTP-II grafts were found significantly higher from respective figures of the GVC grafts. GVC grafts of 3- and 4-weeks could attain 33.04 ± 2.32 and 32.15 ± 3.46 % recoveries respectively while the figures turned out to be 43.04 (± 2.84) and 42.29 (± 2.25) for GTP-I and GTP-II grafts representing 3- and 4-week stages, respectively.

Nucleic acids and total protein contents

The average values of DNA, RNA and total protein contents within the control and gonadectomized grafts are shown in Table I. In first week, the DNA contents showed a significant decrease within the G grafts, as compared with the control grafts. Thereafter, from the 2nd week onwards there were no significant differences in the DNA contents of the experimental and control grafts (Table I). The EDL muscle transplants of G rats showed significantly less RNA contents than the respective control values throughout the study period. The total protein contents of the EDL grafts from the G rats also remained significantly lower than the values for the control regenerates (Table I).

Table I: **DNA, RNA (μg/100 mg of tissue) and total protein contents (mg/100 mg of tissue) of the EDL muscle grafts in the control and gonadectomized rats.**

Type of Graft	STAGES OF REGENERATION (Weeks)			
	1	2	3	4
Control-DNA	193.95[a] ±26.04 (3)	205.52 ±25.23 (4)	159.77 ±28.12 (3)	124.40 ±16.04 (3)
Gonadectomized-DNA	86.40* ±5.07 (3)	170.33 ±29.48 (3)	175.91 ±17.63 (3)	172.42 ±20.26 (4)
Control-RNA	1031.07 ±137.76 (3)	1568.62 ±99.71 (4)	1378.24 ±136.76 (3)	1255.41 ±104.72 (4)
Gonadectomized-RNA	545.51* ±56.37 (3)	1073.31* ±101.64 (3)	665.91** ±36.85 (3)	857.80* ±106.91 (4)
Control-protein	10.57 ±0.39 (3)	11.92 ±1.29 (4)	14.99 ±0.63 (3)	13.68 ±0.69 (4)
Gonadectomized-protein	8.52* ±0.26 (3)	7.24* ±0.96 (3)	7.31*** ±0.44 (4)	8.24** ±1.22 (4)

[a]Mean ± SEM; asterisks show significant difference. *=P<0.05; **=P<0.01; ***=P<0.001. Number in parenthesis indicates sample size (Student's 't' test).

Table II: **DNA, RNA (μg/100 mg of tissue) and total protein contents (mg/100 mg of tissue) of the EDL muscle grafts in the GVC, GTP-I and GTP-II rats.**

Type of Graft	STAGES OF REGENERATION (Weeks)			
	1	2	3	4
GVC/DNA	161.75[a] ±12.57 (4)	141.95 ±2.66 (3)	154.79 ±10.76 (3)	181.74 ±11.29 (3)
GTP-I/DNA	168.64 ±15.44 (3)	138.25 ±3.34 (3)	161.39 ±5.13 (3)	177.61 ±10.50 (3)
GTP-II/DNA	130.36 ±12.22 (5)	186.60**,†† ±9.46 (3)	160.63 ±8.08 (4)	117.55**,†† ±7.61 (4)
GVC/RNA	801.31 ±128.71 (3)	983.11 ±85.31 (3)	785.98 ±53.35 (3)	1046.00 ±95.92 (3)
GTP-I/RNA	568.10 ±73.16 (3)	1070.86 ±183.41 (3)	847.00 ±37.51 (4)	1415.96* ±101.80 (4)
GTP-II/RNA	571.66 ±35.59 (4)	1010.24 ±99.37 (5)	859.66 ±32.41 (4)	1446.33* ±110.00 (4)
GVC/protein	7.64 ±0.46 (4)	8.24 ±0.34 (3)	8.14 ±0.38 (4)	5.85 ±0.29 (3)
GTP-I/protein	6.18 ±0.41 (3)	8.57 ±0.64 (4)	8.73 ±0.48 (4)	14.37*** ±0.86 (4)
GTP-II/protein	6.77 ±0.45 (4)	7.97 ±0.27 (5)	8.03 ±0.71 (4)	10.28**,†† ±0.99 (4)

[a]Mean ± SEM; asterisks are significantly different from respective controls. Significant differences between values of GTP-I and GTP-II grafts are indicated by clubs: *=P<0.05; ††**=P<0.01; ***=P<0.001. Number in parenthesis indicates the sample size.

GVC= gonadectomized plus vehicle injected rats; GTP= gonadectomized and testosterone propionate administered rats.

DNA contents of GTP-I transplants remained statistically unaltered throughout the study period. However, 2-week old EDL muscle transplants in GTP-II rats showed a significant increase followed by a decrease at 4-week stage (Table II). A significant

elevation of about 35% and 38% in RNA contents was observed in GTP-I and GTP-II regenerates, respectively at 4-week stage (Table II). For remaining periods of the study there was no change between the RNA contents of GVC, GTP-I and GTP-II grafts. Regarding total protein contents the data resembles closely with RNA distribution-pattern. The protein contents remained, statistically speaking, unaltered in three types of the transplants for first three sample periods. At 4-week stage the hormone-treated EDL muscle grafts did show significant increase of about 146% and 75% for GTP-I and GTP-II types, respectively in their total protein contents compared to the androgen deprived grafts. The 4-week old GTP-I grafts also contained significantly higher amounts of total protein contents than the GTP-II transplants (Table II).

DISCUSSION

At the end of experimental period, lower % recovery in weight of muscle regenerates in gonadectomized and the higher values in case of testosterone propionate replaced rats as compared to uncastracted and androgen-deprived controls, respectively, indicated that the EDL muscle do respond to presence/ absence of the hormone while regenerating following orthotopic transplantation. The biochemical analyses of these regenerates confirmed this notion. In the absence of androgen the muscle grafts failed to develop the biochemical determinants to the level attained by transplants in uncastrated rats. Significantly lower DNA contents in the androgen-deprived grafts in the initial stage of regeneration is an indicative of lower proliferative activity of satellite cells. As satellite cells are the source of myoblastic cells that give rise to the formation of regenerated muscle fibres within an injured muscle (Snow, 1977a,b; Carlson and Faulkner, 1983; Schultz *et al.*, 1986; Roberts *et al.*, 1989; Schultz, 1989) and their proliferation has been reported to cause DNA increase in growing rats' muscles (Moss and Leblond, 1971; Beermann *et al.*, 1983). Situations of other parameters had not been different from the DNA pattern. These biochemical results support earlier histological and morphometric reports indicating that in the absence of androgens orthotopically transplanted EDL muscle grafts undergo considerable atrophy (Mufti and Qazi, 1987; Qazi and Mufti, 1989).

On the other hand, EDL muscle grafts, in the testosterone propionate replaced rats developed better than the androgen-deprived control animals (GVC), as envisaged by their significantly higher RNA and total protein contents at 4-week stage. Reduced amount of DNA contents in 4-week GTP-II grafts is attributable to the hypertrophied nature of the regenerated muscle fibres in these regenerates (Qazi, 1995), since less number of myonuclei per unit fresh weight of the tissue were sampled. Decline in DNA concentration in growing muscle has been shown to corresponds with the initiation of fibre hypertrophy (Wigmore and Stickland, 1983).

Previously only a select group of skeletal muscles was considered sensitive to androgens; during regeneration and development. The levator ani muscle in rats was an example of such muscles which retained its sensitivity to androgens while undergoing regeneration following various kinds of trauma such as mincing (Carlson *et al.*, 1979). Max *et al.* (1981) have shown that by day 3 in regenerating rat levator ani muscle, after a crush lesion, androgen binding decreased to 25% of control values. This decrease was

followed by a 4-5 times increase in hormone binding, which attained control values by day 7 after crush. These workers have concluded that synthesis of the androgen receptors may occur after the fusion of myoblasts and during the differentiation of myotubes into cross-striated muscle fibres. Later on, Mufti (1985) also showed that formation of androgen receptor ensues within 2-3 days post-crush in the regenerating myoblasts of rat levator ani muscle. The ligand-receptor complexes were found both within the nuclei and the sarcoplasm indicating their possible role in regulating nuclear activity. In conclusion, this information is new, in the sense that EDL muscle which is a fast contracting muscle, not known to be specifically sensitive to androgens, is seen to respond negatively to the absence of testosterone and shows a positive response when supplied with exogenous testosterone propionate, while regenerating following the transplantation. These observations suggest that most of the skeletal muscle in mammals may be sensitive to androgens rather than a few, considered previously. Many more muscles, especially different kinds of muscles, slow and fast, will have to be tested before to generalize the statement.

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(Received: September 16, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 127-137, 1997

MALATHION INDUCED HAEMATOXICITY IN CHICKS OF FOWL, *GALLUS DOMESTICUS*

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Abstract: An organophosphate insecticide, malathion was administered orally to chicks in three different doses *i.e.*, 600, 400 and 250 mg/kg body wt./day for the total periods of 40 hours, 12 days and 4 weeks, respectively. The blood samples were collected following stipulated periods of 5, 10, 20 and 40 hours duration in short term experiment, at 3, 6 and 12 days in 1st long term experiment and at 2, 3 and 4 weeks in 2nd long term experiment and used for various haematological studies.
In short term experiment, significant changes were found in MCV (15% and 10% decrease) and MCHC (13.9% and 14.1% increase) at 20 and 40 hours treatments. In 1st long term experiment the Hb increased by 16%, 12% and 21% at 3, 6 and 12 days. Similar rise was also found in 2nd long term experiment. MCHC increased at 12 days (20%) in 400 mg dose and at 3 and 4 week (16 and 13%, respectively) in 250 mg dose level. The WBC count showed persistant increase in all three treatments.

Key words: Organophosphate insecticide, phosphoro-dithioate, haematology, blood morphology, haematological indices.

INTRODUCTION

The extensive use of insecticides although has lessened the human sufferings by reducing insect-borne diseases and destruction of crops by controlling the pest population but at the same time resulted in wide-spread chemical contamination of the environment. The residues of these insecticides and their derivatives persist in various components of the environment and move towards higher tiers of the food chain through biological magnification (Mugambi *et al.*, 1989; Clavijo *et al.*, 1996; Norman, 1996; Parrilla and Vidal, 1996) and have been found hazardous to animals as well as other environmental systems (Grether *et al.*, 1987; Ali *et al.*, 1988; Ali and Shakoori, 1988, 1990,1996; Shakoori *et al.*, 1988; Singh and Kavadia, 1989; Shirasaka and Konno, 1990; Jianmongkol *et al.*, 1996; Stephens *et al.*, 1996). In recent years the use of insecticides has greatly increased (Roberts, 1996), partly to boost the agricultural output and partly due to development of resistance in insects (Collar and Hink, 1987; Tang Chiong *et al.*, 1989; Bull and Pryor, 1990; Picollo De Villar *et al.*, 1990; Subramanyam and Harein, 1990; Dunkov *et al.*, 1996; Parker *et al.*, 1996; Taylor and Feyereisen, 1996., Berger and Sultatos, 1997).

Amongst these insecticides, organophosphates (OP), occupy a significant place, as far as their usage and role in the environment is concerned. Malathion is one of the most

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widely used OP insecticide against pests of crops, vegetables, fruits and stored grains (Rao *et al.*, 1989; Tang Chiong *et al.*, 1989; Bull and Pryor, 1990; Subramanyam and Harein, 1990). It is also used to eradicate the pests of public health importance. In Pakistan, it has been used against mosquitoes to control malaria by the Health Department.

Malathion is highly toxic to insects and other pests (Kao and Tzeng, 1992; Chakraborti *et al.*, 1993; Al-Shatti *et al.*, 1997) but it is considered one of the least toxic pesticide for nontarget animals (Ali and Shakoori, 1981; Husain *et al.*, 1987; Ansari *et al.*, 1987; Singh and Kavadia, 1989; Thathoo and Prasad, 1989). Poultry may be exposed to malathion and other organophosphate pesticides as these are directly applied to the skin of chicks and other birds beneath the feathers for the prevention of various parasitic infestations (Devaney *et al.*, 1982; McOrist, 1983; Pass and Jue-Sue, 1983). Moreover, poultry farms also require periodical spray to control various vector insects, the factor which is an indirect source of pesticide exposure (pass and Jue-Sue, 1983; Tang Chiong *et al.*, 1989).

Although a lot of work on different aspects of organophosphates and more specifically malathion, toxicity is available in literature, on fish, mammals and various other animals (Mukhopadhyay and Dehadrai, 1978; Paul *et al.*, 1979; Ali and Shakoori, 1981; Singh *et al.*, 1984; Mala, 1987; Richmond and Dutta, 1988; Thathoo and Prasad, 1989; Scarpato *et al.*, 1996; Hughes *et al.*, 1997), very few studies are found as far as the aves and poultry is concerned (Gupta and Paul, 1977; Gromysz-kalkowska *et al.*, 1981; Goyal *et al.*, 1986; Johnston *et al.*, 1994). Only sporadic reports on some aspects of malathion toxicity in poultry, however, exist in literature (Baron and Jhonson, 1964; Varshneya *et al.*, 1988; Rao *et al.*, 1989), while most studies are concerned with the esterase and cholinesterase inhibition (Thompson *et al.*, 1991; Busby *et al.*, 1991; Yadava *et al.*, 1991; Leons *et al.*, 1996), detailed biochemical and metabolic studies are scarce.

In the present report, the effects of malathion on the blood cellular parameters (haematology) in the chicks of *Gallus domesticus* is being reported.

MATERIALS AND METHODS

Animals and their maintenance

The broiler chicks of *Gallus domesticus* were purchased from Hybred (Pakistan) Ltd. Lahore. They were placed in cages of 5 cubic feet size, in the animal house of Zoology Department at 25 ± 1.5°C temperature. During this period, the chicks were fed on commercial poultry feed. The feed and water was provided to the chicks *ad libitum*.

Insecticide used and its administration

Toxicant used for this study was organophosphate (OP) compound,malathion [o.o-dimethyl-S-(1,2-dicarboxy ethyl) phosphoro-dithioate], 57 EC, purchased from Jaafer

Brothers (Private) Ltd., Lahore. The insecticide was administered to chicks orally after proper dilution with water.

Three sublethal doses (one strong and two weak) of malathion were used for the present series of experiments. A strong dose of malathion was administered to chicks @ 600 mg/kg body weight once and its effects were observed for a total period of 40 hours short-term experiment. Two different concentrations were used for two long term experiments. In 1st long term experiment, the chicks were administered with 400 mg of malathion/kg body wt./day for 12 days, while in the 2nd experiment, malathion was given @ 250 mg/kg body wt./day for a total period of 4 weeks.

Experimental plan

In short term experiment, a group of twenty chicks of same age group and size were administered with malathion @ 600 mg/kg body weight once, for the total period of 40 hours. The blood samples were collected from a group of four birds, following stipulated periods of 5, 10, 20 and 40 hours. Another group of four animals, processed similarly, except the insecticide treatment, was used for control experiment. For 1st long term experiment twenty seven chicks were administered with 400 mg of toxicant/kg body wt./day for a total period of 12 days. A group of 4 birds were disected at the interval of 3, 6 and 12 days. In the 2nd long term experiment, fifteen chicks were administered with malathion @ 250 mg/kg body wt./day for the total duration of 4 weeks. A group of 4-5 animals were dissected for sampling at 2nd, 3rd and 4th week. A group of 3-4 chicks processed similarly, except insecticide treatment, was used as control with each treated group. In all above experiments, the blood samples were collected after various stipulated periods, in small glass vials containing EDTA as an anticoagulent for haematological studies.

Methodolog used

The anticoagulent containing blood samples were used for the estimation of haemoglobin (Hb) contents according to Van-Kampan and Zijlstra (1961), packed cell volume (PCV = haematocrit) according to microhaematocrit method of Strumia *et al.* (1954), red blood cell (RBC) counts, white blood cell (WBC) counts according to routine clinical methods. These values were then utilized for calculating the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) according to the formulae mentioned in Dacie and Lewis (1977).

RESULTS

Malathion treatment as strong (600 mg/kg body wt. once) and week doses (400 mg and 250 mg/kg body wt./day) for the total duration of 40 hours, 12 days and 4 weeks, respectively, did not induce any severe alteration in haematological components of chicks (Tables I-III and Figs.1-3).

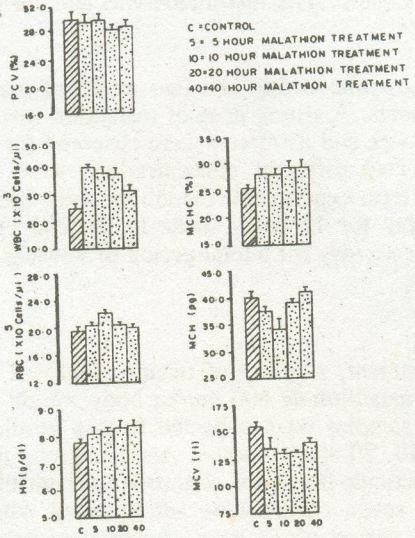

Fig. 1: **Effects of malathion (600 mg/kg body wt.) administered for a total duration of 40 hours on some blood parameters of chick. For abbreviations see Table I.**

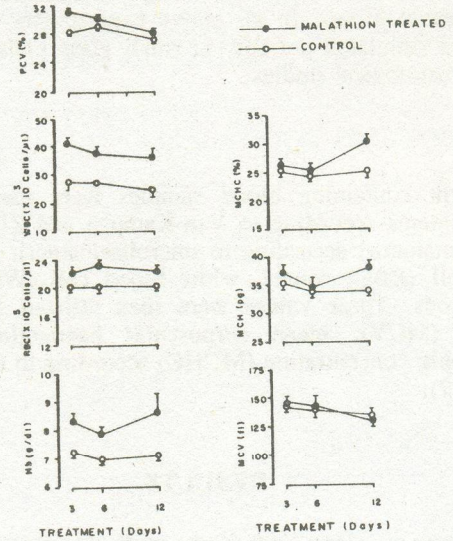

Fig. 2: **Effects of malathion (600 mg/kg body wt.) administered for a total duration of 12 days on some blood parameters of chick. For abbreviations see Table I.**

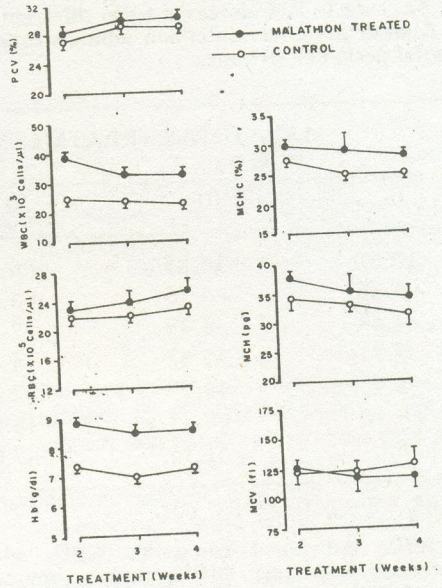

Fig. 3: **Effects of malathion (600 mg/kg body wt.) administered for a total duration of 4 weeks on some blood parameters of chick. For abbreviations see Table I.**

The Hb content, RBC count, PCV and MCH remained disturbed in short term experiment. The significant changes were found in MCV and MCHC which showed 14.65 and 9.90% decrease (MCV) and 14.10 and 13.90% increased (MCHC) at 20 and 40 hours of malathion feeding, respectively. The more pronounced effect was observed in WBC count which was increased 58% within 5 hours of insecticide feeding and retained almost same pattern until 20 hour. The count shot down more than 50% when the treatment was extended for another 20 hours (Table I; Fig.1).

During long term studies the most severely affected parameters were Hb and WBC count. The Hb content shot up by 16%, 12% and 21% in 1st long term I experiment (Table II; Fig.2) and 19%, 14% and 18% in 2nd long term experiment (Tables III; Fig.3). The uninterrupted malathion treatment for 4 weeks caused significant change in WBC count which showed 46%, 41%, 49% rise during 1st long term experiment at 3, 6 and 12 days and 52%, 39% and 40% rise during the 2nd long term experiment at 2, 3 and 4 week treatments, respectively.

In addition, PCV and MCHC also increased by 10% and 20% at 3 and 12 days, respectively after insecticide feeding in 1st long term experiment (Table II; Fig.2). The significant increase of 16% and 14% was found in MCHC in long term II experiment at 3 and 4 week of pesticide feeding. The RBC, MCV and MCH in both long term experiments and PCV in long term experiment remained undisturbed.

Table I: **Percent increase (+) or decrease (-) in different blood components of chick (*Gallus domesticus*) after malathion administration (600 mg/kg body weight) for the total period of 40 hours.**

Parameters[a]	MALATHION TREATMENT (HOURS)			
	5	10	20	40
RBC	+10.81	+15.85	+9.28	+4.49
Hb	+3.86	+4.76	+5.83	+7.41
PCV	-4.48	-4.29	-7.26	-5.65
MCV	-7.71	-5.82	-14.65*	-9.90*
MCH	-5.95	-14.77	-2.84	+2.88
MCHC	+9.10	+8.21	+14.07*	+13.88**
WBC	+58.0***	+51.15***	+47.02**	+22.72*

Student's `t' test, *P<0.05; **P<0.01; ***P<.001.

[a]**Abbreviations used:** RBC, red blood corpuscle; WBC, white blood corpuscle; Hb, haemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin content.

Table II: **Percent increase (+) or decrease (-) in different blood parameters of chick (*Gallus domesticus*) after malathion administration (400 mg/kg body weight/day) for the total period of 12 days.**

Parameters[a]	MALATHION TREATMENT (DAYS)		
	3	6	12
RBC	+11.26	+11.69	+10.08
Hb	+16.05**	+12.15*	+21.06***
PCV	+10.24*	+4.88	+0.88
MCV	+0.40	+01.12	-8.27
MCH	+5.0	+0.43	+10.62
MCHC	+5.06	+6.98	+20.06*
WBC	+46.37**	+41.24*	+48.62**

Student's `t' test, *P<0.05; **P<0.01; ***P<0.001.

[a]For abbreviations, see Table I.

Table III: Percent increase (+) or decrease (-) in different blood components of chick (*Gallus domesticus*) after malathion administration (250 mg/kg body weight/day) for the total period of 4 weeks.

Parameters[a]	MALATHION TREATMENT (WEEKS)		
	2	3	4
RBC	+6.10	+9.41	+7.35
Hb	+18.69**	+14.23*	+17.95***
PCV	+5.97	+1.13	+4.17
MCV	+0.13	-9.21	-3.67
MCH	+11.24	+5.83	+9.34
MCHC	+11.13	+15.93*	+13.39*
WBC	+52.20**	+38.94*	+39.92*

Student's `t' test, *P<0.05; **P<0.01; ***P<0.001.

[a]For Abbreviations, see Table I.

DISCUSSION

Malathion treatment to chicks induced some moderate type of changes when the data of three experiments, with different doses and durations, was compared with other organophosphate and organochlorine insecticides. The changes were more prominent in 1st and 2nd long term experiments. The haematological components which showed prominent alteration were Hb, MCHC and WBC count which showed significant increase in both long term experiments. All other parameters tested, remained unchanged during these treatments, except PCV which was increased at 3 day treatment only and normalized in the remaining duration.

Malathion haematoxicity was also reported in rabbits when administered @ 95 mg/kg body weight/day for 15 month duration (Ali and Shakoori, 1981). The Hb content, RBC count and PCV showed prominent decrease durinng the study while MCV increased. These findings showed that rabbits are more susceptible to malathion. However, long term (15 months) feeding may be the reason for relatively greater toxicity of malathion in rabbits at low doses when compared with chicks in the present study. In another study Shakoori *et al.* (1988) showed unchanged Hb with decrease in RBC and PVC in rats treated with pyrethroid insecticide, cypermethrin @ 420 mg/kg/day for 6 months. Other studies from this laboratory with organochlorine insecticides resulted in greater toxicity when compared with malathion (Ali and Shakoori, 1990, 1994). Contrary to this, the results in the present report with malathion indicated some-what stimulatory effect on haematopoietic system especially haemoglobin biosynthesis, with the corresponding increase in MCHC because RBC count remained undisturbed. During short term experiment, decrease in MCV and rise in MCH was an indication of fairly high compactness of the Hb in the RBC's. This may also be ascribed

to increased biosynthesis of Hb. Similar changes in MCHC were also noticed with cypermethrin in rats (Shakoori *et al.*, 1988). It has been reported in birds that hypoxia and development of dehydration may be the reason for increased Hb, RBC, MCH and PCV which is a protective mechanism to compensate the decreased uptake of oxygen under toxic stress (Coles, 1986; Campbell, 1988). These findings were also confirmed by Natarajan (1984) working with metasystox on fish. According to this report the inhibition of succinic dehydrogenase and tissue respiration due to oxygen depletion, strengthen this assumption. Although in this report, oxidative enzymes were not studied but on the basis of above report it is suspected that malathion may develop hypoxic conditions in chicks. However, this thing needs further experimentation in chicks with malathion.

The WBC count is another important haematological component, which exhibited significant alteration following malathion administration in all treatments (40 hours, 12 days and 4 weeks). Rise in WBC count is a typical response of vertebrates in case of toxic insult with a variety of toxicants. There are several reports from this laboratory which show increased WBC after treatment with different pesticides (Ali and Shakoori, 1990, 1994). Similarly Dikshith *et al.* (1980) working with phosphamidon in rabbits, Gromysz-Kalkowska *et al.* (1981) working with trichlorfon in quails and Gromysz-Kalkowska and Szubartowska (1986) working with trichlorfon in frog also showed increase in WBC which is a defensive response of the organism against foreign toxic invasion. In conclusion, increase in WBC count may be due to damaging action of malathion on blood cells and tissues which are required to counter and detoxify the effects of this toxicant and to phagosatize the dead or necrotic tissues.

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(Received: April 10, 1997)

Punjab Univ. J. Zool., Vol. 12, pp. 139-150, 1997

SOME HAEMATOLOGICAL CHANGES PRODUCED IN RABBITS AFTER INTRAVENOUS INOCULATION OF UNILOCULAR HYDATID CYST FLUID OF SHEEP ORIGIN

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Abstract: Experimental rabbits were inoculated with hydatid cyst fluid (HCF) of sheep origin to determine its toxicity by hematological changes in rabbits (*Oryctolagus cuniculus*). Five groups of animals were administered with crude low dose (CLD), filtered low dose (FLD), crude medium dose (CMD), filtered medium dose (FMD) and high dose (HD) of HCF and one control group given distilled water with same protocol. Blood samples were collected after every 14 days and different haematological analyses were made. An increasing trend of WBC count in all the groups was noted except for HD group in which decrease was noted. It was noted that RBC count shows initial increase in low dose groups but ultimately decreased resulting in anemic condition. Under both low dose treatments, PCV increased but decreased in medium and high dose groups. Haemoglobin (Hb) also decreased with the corresponding increase in the dose of HCF. MCV increased in low dose groups but decreased in medium dose and HD groups. There was general decrease in mean corpuscular haemoglobin and mean corpuscular haemoglobin content. ESR however, showed increasing trend with the increase in HCF dose. The interesting feature was the development of tolerance against HCF in almost all the cases. This is probably responsible for this disease to be of non dramatic type as for as symptoms are concerned in natural infection. It was further noted that the low dose groups reflected macrocytic hypochromic anemia where as medium dose and HD group reflected microcytic hypochromic anemia.

Key words: Experimental hydatidosis, haematological changes, rabbit.

INTRODUCTION

Bidirectional movement of different materials through the hydatid cyst has been given consideration by a number of workers. *In vitro* permeability studies on hydatid cysts of *Echinococcus granulosus*, obtained from rodents by experimental secondary infection, have shown protein uptake by cysts (Coltorti and Varela-Diaz, 1975). Hustead and Williams (1977) stated the occurrence of cyclic uptake activity. The permeability of secondary *E. granulosus* hydatid cysts to water, sodium and chloride ions has been determined *in vitro* under steady state conditions by Rotunno *et al.* (1974). The HCF have some altering effects on the hematology of experimental animals. There was increased leukocyte count, decreased erythrocytic count and haemoglobin content as reported by Amnizhanov (1977) in sheep experimentally infected with *E. granulosus*. Hinz and Gehring (1985) studied the mice

0079-8045/97/0001-0137 $ 03.00/0

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infected with *E. multilocularis* and found an increase in lactation, red blood cell count, haemoglobin content, packed cell volume and neutrophils. In contrast, *Meriones unguiculatus* (Mangolian gerbils) when intraperitoneally inoculated with *E. multilocularis* showed a decrease in packed cell volume after 2 and 14 week of inoculation (Kroeze and Tanner, 1986). Similarly a decrease in packed cell volume, erythrocyte number and haemoglobin content was noted after two week in mice experimentally infected with *E. multilocularis* (Hinz and Gehring, 1987). Wangoo *et al.*, (1989) observed a significant increase in phagocytic activity of blood monocytes in albino mice in later stages of infection with *E. granulosus*. El-Gindy *et al.* (1990) intraperitoneally infected five groups of 60 Swiss albino mice with 2000 aseptic normal (control group) and gamma-irradiated (experimental group) *E. granulosus* larvae. Hematological studies showed a marked leukocytosis, a progressive increase in the average percentage of eosinophils as well as a large successive decrease in haemoglobin concentration throughout the time of infection in controls, compared to the experimental groups. Alterations in various haematological parameters due to hydatid cyst fluid have also been reported by Tanveer *et al.* (1998, 1998a) in rabbits. Present work deals with some hematological changes in rabbits after inoculating intravenous hydatid cyst fluid both in filtered and crude forms.

MATERIALS AND METHOD

Rabbits (*Oryctolagus cuniculus*) maintained in the optional conditions of animal house were fed on seasonal green fodder and tap water *ad libitum*. The feeders and drinkers were daily washed, with concentrated $KMnO_4$ solution to disinfect them. Rabbits were acclimatized for fifteen days to the conditions of animal house prior to experimentation. They were divided into five experimental groups depending upon the type of treatment and one control group (n=3) given distilled water with same protocole was run for each experimental group like (*a*) crude low dose (CLD), (*b*) filtered low dose (FLD), (*c*) crude medium dose (CMD), (*d*) filtered medium dose (FMD) and (*e*) high dose (HD) groups. Dose of HCF to each group was given through intravenous injections according to following schedule.

Hydatid cyst fluid (HCF) aspirated from infected sheep harboring cysts was transferred into air-tight sterile glass vials and placed at 4°C. Required quantity of fluid was filtered through Whatmann filter paper No.1. As slaughtering is legally prohibited in Pakistan on every Tuesday and Wednesday, during these days refrigerated HCF was administered to the rabbits. Flame cell activity of the protoscoleces was always observed before giving fresh and stored HCF. Blood samples were fortnightly pooled from control and experimental groups. About 2 ml of blood was taken from each rabbit in the test tubes containing 4 mg of anticoagulant, disodium ethylene diamine tetra acetic acid (EDTA) (E. Merck Germany). The tubes were gently rotated for about five minutes to mix EDTA with blood. Different hematological parameters like, white blood cell counts (WBC), red blood cell counts (RBC) both according to Dacie and Lewis (1991), packed cell volume (Strumia *et. al.*, 1954), haemoglobin content (VanKampen and Zijlstra 1961) and ESR were determined by Westergren's method (Swarup *et al.*, 1986) and hematological indices like, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), were

calculated according to Dacie and Lewis (1991). The data was analyzed by "Students "t" test. according to Steel and Torrie (1981).

Duration (days)	GROUPS			
	CLD FLD (ml/day)	CMD FMD (ml/day)	HD (ml/day) 1st week	HD (ml/day) 2nd week
1-14	10	0.1	0.1	0.15
15-28	20	0.15	0.2	0.25
29-42	30	0.2	0.3	0.35
43-56	40	0.25	0.4	0.45
57-70	50	0.3	0.5	0.55
71-84	50	0.3	0.55	0.55
85-98	50	0.3	0.55	0.55

Footnote: In each group, n=7.

For abbreviations, see Figure 1.

RESULTS AND DISCUSSIONS

Fig.1 showed that in control group WBC counts remained almost unchanged through out the study period. While they increased in the CLD and CMD group non-significantly. An increase ($P<0.05$) in WBC count of FLD group was noted (Fig.1). The increase in WBC count of CMD group was found significant ($P<0.05$) after 70, 84 and 98 days of FHCF administration. HD group showed decreasing trend in WBC count. The decrease in WBC count in HD group is not in accordance with the other groups. The decrease was however, statistically significant ($P<0.05$) after 70 days of FHCF administration. White blood cells or leukocytes constitute a very important mechanism in the animal body in combating different infections and other invading toxic agents like trauma, germs and various foreign particles. They destroy invading agents either by the process of phagocytosis or they form antibodies and sensitized lymphocytes which may destroy the invader (Gusto, 1991). Subsequent to the tissue damage, either caused by bacteria, trauma, chemicals, heat or any other phenomenon (like inoculation of HCF), multiple substances are released by the injured cells. That results due to the migration of large number of granulocytes and monocytes into the tissue with an ultimate increased number of white blood cells in the experimental groups (Boyd, 1970). During present investigation elevated WBC count in CLD, FLD, CMD and FMD groups is probably due to the increase of phagocytes and/or eosinophils to combat with incoming foreign proteins and allergic threat evoked by crude and filtered HCF. WBC increase was more pronounced in both of the crude groups than the filtered. That was probably due to the presence of insoluble protein sub-units like degraded protoscoleces, brood capsules, fragmented germinal membrane etc., in crude hydatid cyst fluid that have been removed from FHCF during filtration. Increase in WBC counts can also be

attributed to tissue the damage (Eastham, 1985) which may be caused by the proteolytic enzymes and/or toxins present in HCF. Present results are in accordance with Aminzhanov (1977) who observed an increase in leukocytic count in infected sheep with *E. granulosus*. Similar increase in phagocytic activity of monocytes was also studied by Wangoo *et al.* (1989) and Alkarmi and Behbehani (1989) in albino mice infected with *E. granulosus*. Increased WBC counts in mice infected with *E. multilocularis* was also noted by Hinz and Gehring (1987) and Alkarmi and Ali Khan (1989). In the blood tissue granulocytes and agranulocytes are most important elements for defense against invading micro-organisms. Their power to attack foreign bodies depends mostly upon their motility and desire for ingestion of solid particles. The latter action is termed as phagocytosis. These two types of WBC are free lancing among the body cells (Charles and Norman, 1966). It was also noted during present studies that the toxic and pathological impact of HCF upon blood and tissue was parallel in many of the case to the poisonous properties of insecticides, pesticides, herbicides, and with exo and endotoxins. Because HCF and insecticides disturbed the blood biochemistry and physiology in almost similar way, hence the toxicity of HCF has been compared to such compounds. On the other hand leukocytic count in HD group showed constant decrease as compared to elevated WBC count of other four groups. This may be due to higher concentration of toxins in HCF administered under High Dose dose, which may inhibit the synthesis or increase the destruction of WBCs due to the presence of enzymes (McManus and Smyth, 1982) and toxins (Wangoo *et al.*, 1987). Results of all the experimental groups except High Dose group showed normal values of WBC count probably due to the tolerance developed by the animal against toxins and/or antigens.

In normal body conditions the population of red cells and the concentration of haemoglobin in the blood are kept at normal level by nice balance between the population of newly formed and the old erythrocytes to be destroyed (Gusto, 1991). Both CLD and FLD groups showed increase in RBC count up to 28 days of HCF administration. This trend indicated that HCF may trigger RBC and haemoglobin synthesis under low doses and that may be due to interference of HCF in the binding of oxygen to haemoglobin. During present investigation it was noted that increase in RBC count and haemoglobin content did not persist but decreased after 42 days of HCF administration. This is probably due to inhabitation of RBC biosynthesis and/or destruction at high or prolonged dose of HCF which significantly decreased haemoglobin content after 70 days of HCF administration in both CLD ($P<0.01$) and FLD ($P<0.05$) groups. CMD, FMD and HD groups showed reduction in the total erythrocyte count from the start of HCF administration probably due to damage or inhibition of erythrocyte synthesis. However, in the final stages this reduction was prominent in all the experimental groups. This RBC deficiency syndrome is called anemia, which results by rapid loss or slow production of red blood cells. In the present study ultimate reduction noted in RBC count is similar to the findings reported by Aminzhanov (1977) in sheep, infected with *E. granulosus*. Increased RBC breakdown is also responsible for the increased plasma bilirubin (Charles and Norman, 1966). This loss may be due to the direct effect of toxicant on the blood cell or indirectly through its effect on bone marrow, the blood forming tissue (Rajini *et al.*, 1987). It was observed that in many organs, these incoming toxicants are responsible for the development of necrosis (Chaitow *et al.*, 1975) and in such cases histamine was found in these areas (Billewicz-Stankiewicz, 1955) which damage the capillary cell wall and fine veins leading to the blood extravasation (Cown, 1974).

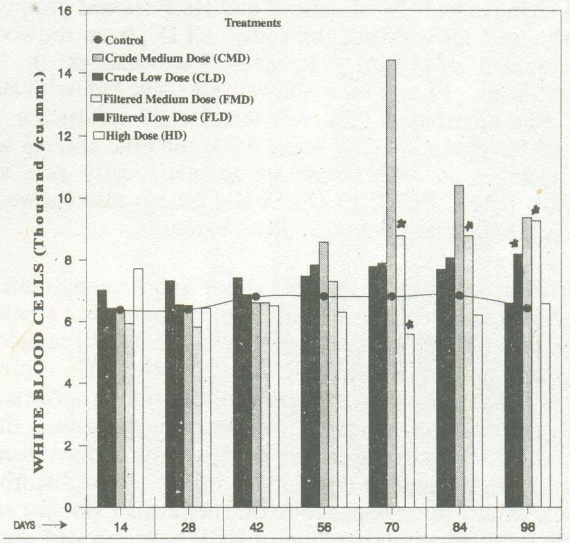

Fig. 1: Changes in WBC count of rabbit after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses. *P < 0.05; **P < 0.01; ***P < 0.01.

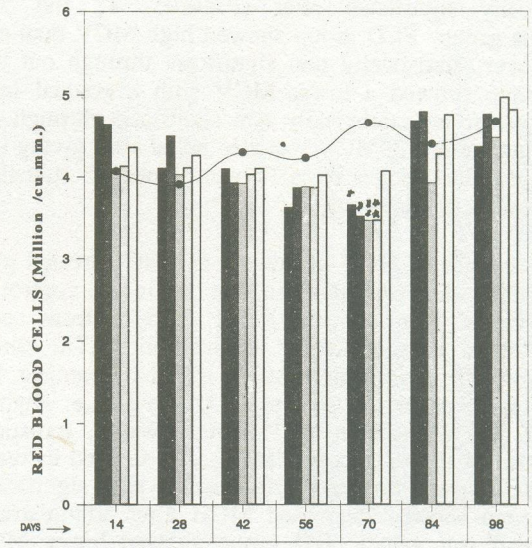

Fig. 2: Changes in RBC count of rabbit after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses. For abbreviations and key to graph, see Fig.1.

Fig.3 showed changes in PCV of control and HCF treated groups. In control group PCV remained unchanged through out the study. CLD group showed increase in PCV initially which decreased afterward. However, the changes in PCV were found statistically non significant. FLD group showed elevated but with decreasing trend in PCV. CLD group. The increase in PCV was statistically significant ($P<0.05$) after 28 days of treatment. CMD group showed lower PCV than the control with corresponding increase. All the changes in PCV were found statistically non significantly when analyzed by Student's 't' test. FMD, FLD and HD groups also showed a non-significant decrease in PCV like CMD group but with lesser extent.

In the present study, reduction in RBC count and haemoglobin content in all the experimental groups was either due to inhibition of haemoglobin synthesizing enzyme or haemolysis that leads to binding of haemoglobin to plasma hepatoglobins. During this process excessive haemoglobin is excreted through urine (Gowenlock *et al.*, 1988). Haemoglobin is partly converted to metahaemoglobin prior to conversion in to bilirubin. In the present investigation the increased bilirubin level showed the enhanced break down of haemoglobin. The ability of certain chemicals to induce haemolytic disease by generation of autoantibodies suggests that similar immunologic disturbances could be the basis of bone marrow injury in response to toxic chemicals (Morgan *et al.*, 1980). In the present investigation repeated administration of HCF have induced anemia in rabbits.

Fig. 4 showed changes in MCV of control and HCF treated groups. It was noted that the values for MCV remained unchanged through out the study in control group. The CLD group showed an increased MCV as compared to their control group, which is highly elevated even from the beginning (14 days) of CHCF treatment. The increase in MCV was statistically significant after ($P<0.05$) 42 and 56 days of CHCF administration in this group. FLD group showed high MCV than control. The changes in MCV were however, statistically non significant through out the treatment in this group. In CMD group showed a lower MCV with a gradual increasing trend. The changes in MCV were found statistically non significant through out the treatment in this group. Decreasing pattern in MCV was also noted after giving intravenous FHCF to FMD and HD groups. The changes in MCV were however, statistically non significant through out the treatment in this group.

Fig.5 showed changes in MCH of control and HCF treated groups. IT was noted that the values of MCH showed minor fluctuations in the control group. CLD group showed initially declined then elevated MCH contents from control Fig.5 showed maximum MCH after 70 days of CHCF administration. The changes in MCH were however, statistically non significant through out the treatment in this group. FLD group followed the same pattern as that of CLD group, i.e. slight elevation in MCH than that of control. The changes in MCH were however, statistically non significant through out the treatment in this group. CMD group showed increasing trend of MCH. The changes in MCH were however, statistically non significant through out the treatment. FMD group showed decreased MCH after administration of FHCF with statistically non significant values. HD group showed lower MCH through out the period of treatment. The decrease in MCH were however, statistically significant ($P<0.01$) after 70 days of treatment.

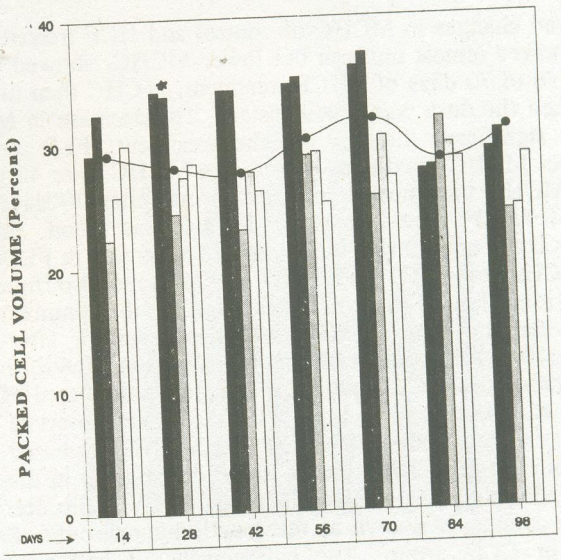

Fig. 3: Changes in packed cell volume of rabbit after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses. For abbreviations and key to graph, see Fig.1.

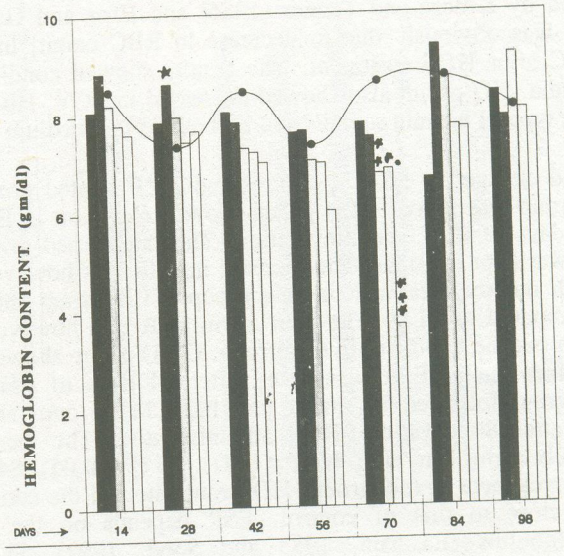

Fig. 4: Changes in haemoglobin content of rabbit after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses. For abbreviations and key to graph, see Fig.1.

Fig.6 showed changes in MCHC of control and HCF treated groups. In the CLD group MCHC showed almost uniform but lower MCHC, showed a maximum decrease in MCHC after 56 to 70 days of CHCF treatment. MCHC then turned towards normal after 70 days when the dose was kept constant. The decrease in MCHC was however, statistically non significant through out the treatment. FLD group showed almost constant but lower MCHC maximum decrease in MCHC after 42 to 56 days of FHCF administration. MCHC then turned towards normal after 70 days at consective three constant doses of FHCF. The decrease in MCHC was found, statistically significant only after 42 ($P<0.01$) and 70 ($P<0.05$) days of treatment in FLD group. Fluctuations noted in the MCHC of CMD and FMD group were also found statistically non-significant. HD group showed low MCHC through out the administration of FHCF. The MCHC raised when the dose of HCF was kept constant after 70 days of FHCF administration ($P<0.01$). Results of present investigation showed that at very low doses of HCF administration (as in CLD and FLD groups) an increased PCV was noted which is in accordance with Hinz and Gehring (1985). It was observed that in FLD group haemoglobin content increased while MCH and MCHC decreased. This showed RBC deficit haemoglobin most probably due to some disturbance in the permeability of the cell membrane. FLD group also showed increased MCV while decreased MCHC. Such type of anemia is commonly known as macrocytic hypochromic. Same type of anemia was also noted in CLD group. The enlargement of erythrocyte may be due to accumulation of fluids after disturbance in cell membrane permeability (Sood, 1992). The situation remained as such in latter stages but ultimately RBC and haemoglobin content decreased in both CLD group and FLD group. This was probably due to the break down of RBC and haemoglobin which resulted into increased bilirubin content (Anwar and Tanveer, 1997). In contrast to both low dose groups the medium dose group showed non-significant decrease in PCV after HCF administration. Similar findings have also been reported by Kroeze and Tanner (1986) and Hinz and Gehring (1987). This decrease in PCV was obviously due to decrease in RBC count, haemoglobin, MCH, MCV and MCHC after HCF treatment. The results showed conditions of microcytic hypochromic anemia. HD group also showed decreased in PCV, Hb, MCV and MCHC representing same type of anemia as indicated by both of the medium dose groups.

Fig.7 showed changes in ESR of control and HCF treated groups. No change in ESR of control group was noted. CLD group showed elevation in ESR with maximum increase after 70 days of CHCF administration. The rate turned towards normal when the dose of HCF was kept constant. The increase in ESR was however, statistically non significant through out the treatment in this group. FLD group showed slightly high ESR values as compared to CLD. The increase in ESR was however, statistically non significant through out the treatment in this group. CMD group showed rapid increase in ESR. Maximum increase in ESR was noted after 84 days of CHCF administration ($P<0.05$). FMD group followed the similar path like CMD group. There was maximum increase in ESR after 84 days of FHCF administration. The increase in ESR was however, statistically significant after 42 ($P<0.01$), 56 ($P<0.05$), 84 ($P<0.01$) and 98 ($P<0.05$) days of treatment in this group. HD group showed the constant but increased ESR which was close to that of control. ESR depends on the concentrations of fibrinogen and globulins (Eastham, 1985 and Sood, 1992). Since HCF contain fibrinogen and globulins (Sanchez and Sanchez, 1971) therefore its administration in the blood of experimental animals is responsible for the increased value of ESR. Comparatively less pronounced increased ESR was noted for CLD group as compared to FLD group. Similarly CMD group showed initially less prominent ESR than FMD

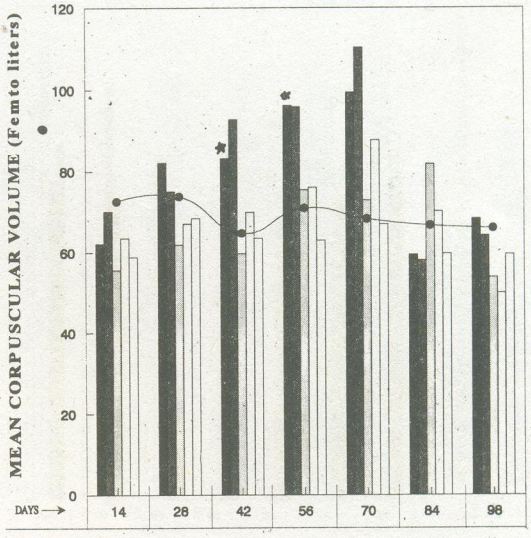

Fig. 5: Changes in mean corpuscular volume of rabbit blood after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses. For abbreviations and key to graph, see Fig.1.

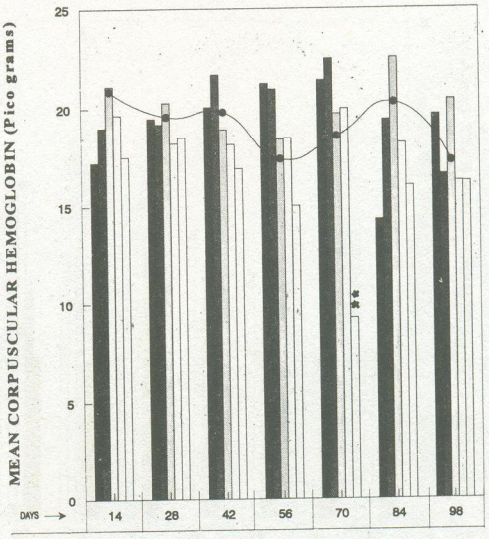

Fig. 6: Changes in mean corpuscular hemoglobin of rabbit blood after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses. For abbreviations and key to graph, see Fig.1.

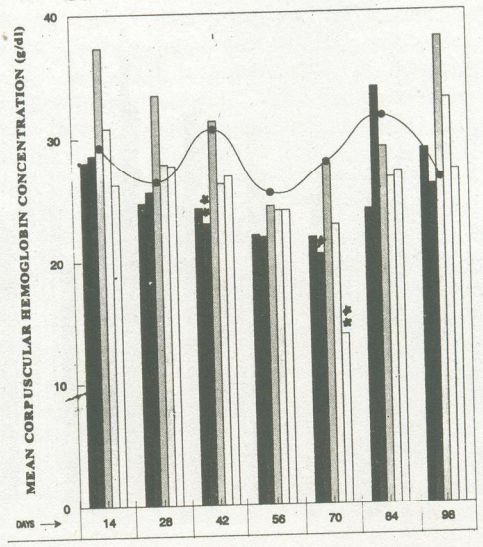

Fig. 7: **Changes in mean corpuscular hemoglobin concentration of rabbit blood after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses. For abbreviations and key to graph, see Fig.1.**

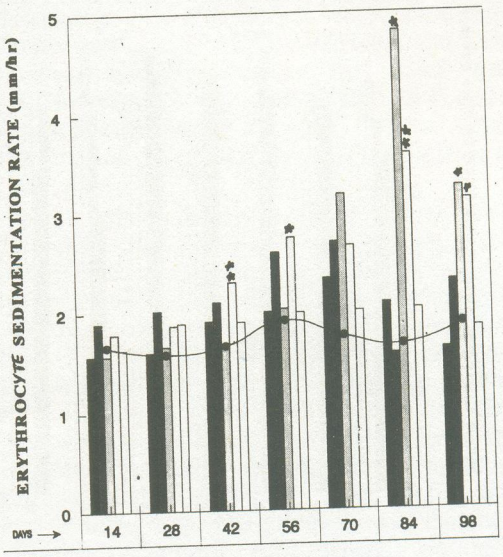

Fig. 8: **Changes in erythrocyte sedimentation rate of rabbit blood after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses. For abbreviations and key to graph, see Fig.1.**

group is probably due to the interference of insoluble proteins, brood capsules, protoscoleces and or their metabolites, which interfere in the rouleaux formation, hence decreasing the ESR value in crude groups. However, after 70 days of continuous administration of CHCF to CMD group, these values increased most probably due to the activity of protoscoleces which were in the course of cyst formation and were absent in FMD groups.

Acknowledgement

Financial aid given by Pakistan Science Foundation, P-PU/Agr (137) is gratefully acknowledged.

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(Received: May 14, 1997)

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