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CONTENTS

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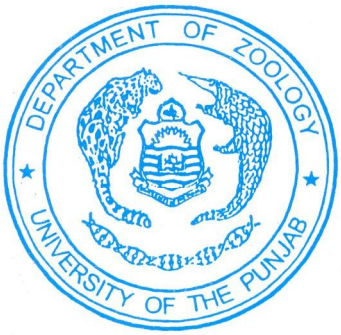

DEPARTMENT OF ZOOLOGY

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PREVALENCE OF *LYMNAEA* SNAILS IN LAHORE DISTRICT AND THEIR ERADICATION BY A MULLUSCICIDE COPPER SULPHATE

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Abstract: The study was designed to assess the pevalence of the *Lymnaea* snails, identification of infected and non-infected snails with intermediate stages of liver fluke and to recommend suitable copper sulphate concentration as molluscicide under laboratory and experimental pond conditions. Prevalence of *Lymnaea* species was found to be 42% and infection of *Lymnaea* snails with intermediate stage of *Fasciola hepatica* ranged between 38% and 69% in five habitats studied during the period from October 1992 to March 1993. Copper sulphate dilution 1 mg/100 ml (10 ppm) was found to be effective as molluscicide and safe for other aquatic fauna and vegetation.

Key words: *Lymnaea, mulluscides*, snails, seasonal variations, *Fasciola hepatica*, *Fasciola gigantica*.

INTRODUCTION

A large number of diseases of man and animals have been reported to be transmitted through different species of snails. Transmission of human trematode infections, including clonorchosis, paragonomosis, intestinal fluke infection and schistosomiasis through snail vectors have been reported (Webb, 1984). Among trematodes, members of Fasciolidae, Dicrociliidae, Paramphistomatidae and Schistostomatidae are transmitted in animals through snail vectors. Of all trematodes of veterinary importance, members of the family Fasciolidae belonging to the genus *Fasciola* are responsible for causing fascioliasis in cattle, buffaloes and sheep. The disease has been reported to be of world wide prevalence. *Fasciola hepatica* and *F. gigantica*, the causative agents of Fasciolosis in livestock are transmitted through different *Lymnaea* species of snails (Soulsby, 1982). *Lymnaea auricularia, L. rufescense* and *L. andersolana* have been reported as fasciolosis transmitter in India (Prasad *et al.*, 1987). Prevalence of *L. rufescens, L. acuminata* and *L. auricularia* have been confirmed in different districts of Sindh by Sarwar (1956). *Lymnaea* species of snails are usually found on decaying floating leaves (42.73%), aquatic submerged vegetation (34.08%), wood-logs and floating objects (16.12%). Lymnaeid snails are rarely found from the bottom of the ponds. Similar findings have been reported from this laboratory (Tanveer *et al.*, 1990).

For the control of any parasite, its life cycle has to be interrupted. For the purpose

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snail (vector) can be eradicated by the use of molluscicides. Some 7000 compounds have been screened as molluscicides (Ritchie and McMullen, 1961). Ivermectin was used as a molluscicide by Okafor (1990). Mius *et al.* (1990) used acetaldehyde and metaldehyde as a molluscicide. Bayluside 70% wettable powder has been applied in large irrigation canal and lakes in Mali by Madsen *et al.* (1986). Copper sulphate has slow solubility and high specific gravity hence it is only toxic to organisms ingesting them on the bottom of water bodies and therefore comparatively harmless to other aquatic life. It is freely available and easily applicable (Anon, 1973; Cheng, 1975).

In the present study prevalence of *Lymnaea* snails, rate of their infection with intermediate stages of liver fluke and their correlation with meteorological conditions in Lahore district was studied. Moreover, study on the efficacy of different concentrations of copper sulphate as molluscicide *in vitro* and *in vivo* was undertaken.

MATERIALS AND METHODS

Collection of snails

One thousand snails were collected fortnightly from five places in Lahore, 200 from each of the National Ravi park (adjacent to river Ravi at Shahdara), Bansi Sagar fish pond (Mustafa-abad, Cantt), Botanical garden pond (University of the Punjab), a pond located in Lawrence garden and Mustafa-abad drain.

Snails were brought to the laboratory for their identification and of intermediate stage of *Fasciola* spp. Identification of snails was done by the details given by Malek and Cheng (1974). After every fifteen days snails were collected and brought to the laboratory. For this purpose the number of dead and alive snails were taken under consideration. The time of collection wasnot considered. While transferring the snails from the acclimated temperature to the room temperature, the snails were first brought to the required temperature slowly within 2 hours, in order to avoid mortalities due to sudden temperature shock.

Identification and maintenance of Lymnaea snails

The snails have thin shell usually with a prominent acute spire and a large, often flaring aperture, varying from horn-color to black, lip acute and simple; tentacles flattened; eggs laid in jelly; radula with unicuspid central tooth, jaws composed of three pieces, one large transversely elongate piece and two, small ones; foot rounded behind. The maintenance of snails was done as suggested in the manual published by MAFF (Ministry of Agriculture, Fisheries and Food, 1986).

Identification of Trematodes

The identification of immature stages of trematodes was done according to the techniques described in MAFF (1986).

Application of molluscicide (Copper sulphate)

Two standard doses of copper sulphate *i.e.*, 1 mg/66 ml suggested by Osterberg (1987) and 1 mg/100 ml ponds located in the College of Veterinary Sciences, Lahore and 4 laboratory aquaria. Comparative efficacy of the two concentrations of $CuSO_4$ was

assessed by the rate of mortality of the snails and safety of other aquatic fauna. Standard temperature ($25 \pm 1.5°C$) with pH 7.4 was tried to and water 3000 ml/2 snails was kept to maintain as a standard habitat in the laboratory as described by Tanveer *et al.* (1989).

Collection of meteorological data

Meteorological data of distt. Lahore was recorded to study the correlation of habitats of snails as well as of intermediate stages of liver fluke under local conditions.

Estimation of water

Volume in liters of the cylinder shaped experimental pond was calculated by the formula $\pi r^2 h$, where r is radius and h is the height ofthe cylinder.

Statistical analysis

Data were analysed by using statistical test formula and the values obtained was compared with the tabulated values under 10 degree of freedom tonote the significant difference for the molluscicide dilutions.

RESULTS AND DISCUSSION

A total of 8000 snails were collected from five places in and around Lahore, which included different genera namely; *Lymnaea, Gyraulus, Physa, Bulinus* and *Oncomelania*. The percentage being, 42.36, 19.11, 12.29, 12.54 and 13.7 respectively. However, out of 8000 snails only 2465 (30.8%) were found alive during whole of the study period.

The study was undertaken during the period from October 1992 to March 1993. It was found that monthwise percentage of *Lymnaea* spp. were 30.8, 16, 15.78, 0, 28.46 and 64.02% from October 1992 to March 1993. Further details have been given in Table I. Monthwise prevalence of snail has been studied by different research workers. Mushtaq (1983) claimed that *Lymnaea* snails were maximum in number when the temperature was warmest and minimum in November when the temperature was coldest. The present findings are in agreement with these studies but different with Hassan *et al.* (1984) who reported the highest prevalence (28.55) of *Lymnaea* snails during the month of January but lowest in June to October, in Fayum province of Egypt. The difference may be due to different ecological conditions in both studies.

Five places were studied during the present work. It was found that from October to March, the prevalence of alive snails in Ravi fish pond was 11% in October and no snails could be found in other months as fish pond became dry. In Mustafa Abad drain 33% in October, 0% in November, December and January, 38.54% in February and 46.23% in March, alive *Lymnaea* spp. were found. In Jinnah garden (experimental *Lymnaea* snails rearing pond) during January and February no snails could be recovered. From Bunsi Sagar fish pond 35.7%, 7.5%, 0.0%, 6.75% and 9.93% whereas from Botanical gardens 13.46%, 8.69%, 12.98%, 0% and 0% alive *Lymnaea* snails were recovered during the months from October to March, respectively. The findings which clearly indicate the lowest prevalence during the winter months. It was noticed that *Lymnaea* spp. disappeared in Ravi Fish Pond due to dryness of pond. The results

Table I: Prevalence of alive snails belonging to different genera collected from various study areas.

		October		November		December		January		February		March		Total	
		T	A	T	A	T	A	T	A	T	A	T	A	T	A
L	No.	804	232	635	57	789	48	330	-	229	80	602	427	3389	844
	%age	40.2	30.8	45.68	16	49.37	15.7	41.25	-	28.62	28.4	50.16	64	42.39	34.69
G	No.	406	178	291	111	245	115	171	66	166	95	250	93	1529	658
	%age	20.3	23.63	18.18	31.15	15.37	37.7	21.37	66	20.75	33.8	20.83	13.8	19.17	26.69
P	No.	175	37	175	74	320	69	63	23	188	36	62	26	983	265
	%age	8.75	4.9	10.93	20.7	120	22.62	7.87	23	23.5	12.81	3.8	3.88	12.28	10.75
B	No.	270	146	229	68	147	65	89	8	83	18	183	69	1003	374
	%age	13.5	19.38	14.37	19.18	9.18	21.31	11.12	8	10.37	6.4	15.4	10.29	12.53	15.17
O	No.	345	160	270	46	99	8	147	3	134	52	101	55	1096	324
	%age	17.25	21.24	16.87	12.93	6.18	2.62	18.37	3	16.75	18.5	8.4	8.2	13.7	13.14
Total		2100	853	1706	456	1800	405	900	200	900	381	1299	770	8100	2565

Abbreviations: L = *Lymnaea*, G = Gyraulus, P = Physa, B = Bullrinus, O = Oncomelania, T = Total No. of snails, A = alive snail.

Note: The percentage was calculated with reference to total numbers collected per month.

substantiated with the findings of Mushtaq (1983) who claimed that *Lymnaea* cannot live long in an environment devoid of water as they are least resistant to desication.

The temperature and humidity are other factors for the survival of *Lymnaea* spp. and other snails, as it has been studied by Tanveer *et al.* (1989). They conclude that the optimal temperature for *Lymnaea* spp. was $26 \pm 1°C$ whereas the minimum lethal temperature noted was 40°C. During December 1992 and January, 1993 the minimum temperature ranged between 9.4°C and 6.7°C, respectively which is detrimental for the survival of snails. That is why, during this period the *Lymnaea* snails could not be found in the ponds. Effect of humidity has also been studied by Mushtaq (1983) who referred that maximum number of snails in the month with least humidity (May) and the minimum number in the month with high humidity were recovered. The humidity levels relating to the recovery of snails are given in Table II.

Six experiments in duplicate were conducted in fish aquaria and experimental pond to observe the effect of copper sulphate dilutions *i.e.*, 1 mg/66 ml of water and 1 mg/100 ml of water. Each experiment consisted of 28 snails of different genera as well as for each dilution. The results showed that 1 mg/66 ml dilution showed mortality dilution of snails between 46.42% and 60.71%. Unpaired t-test showed no significant difference amongst the two dilutions when compared with the tabulated value at 10 degree of freedom at x/2 levels. It was observed that 1 mg/66 ml of dilution was obviously better and effective for the control of snails. However, fingerlings of fish present in the 1 mg/100 ml dilution were not affected at all even up to 72 hours whereas all snails were killed within 22 hours of medication. A lot of work has been undertaken for the use of copper sulphate as molluscicide by number of workers (Webb, 1984).

Table II: Effect of average relative humidity (percent) on the snails population.

	October	November	December	January	February	March
Average Humidity	62	71.5	73.5	64.9	60.0	58.9
Total snails	2000	1600	1800	800	800	1200
Alive snails	753	356	305	100	281	670

Copper sulphate as effective molluscicide has been used by many workers and it has been found that 10 ppm concentration can be used with better safety index and less cost of its treatment.

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SHORT AND LONG TERM TOXICITY OF DDT IN ALBINO RATS: BIOCHEMICAL EFFECTS IN LIVER*

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Abstract: An organochlorine insecticide, DDT was given to rats @ 100 mg, 20 mg and 10 mg/kg body weight orally alongwith the feed in three durations for 48 hours, 15 days and 18 months, respectively. After stipulated periods, animals were dissected, and liver samples were quickly excised and subject to varios biochemical analyses. DDT administration to rats produced significant decrease in body weight gain/day after 9, 12 and 15 days in 15 day experiment and after 18 months in long term experiment. The relative liver weight on the other increased in all treatments. The hepatic gltamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) increased 77 and 35% after 48 hours, respectively. The GOT activity increased (61% and 112% in 15 day and 18 month experiment), while GPT increased 86% during 18 month study. Lactate dehydrogenase (LDH) increased 71, 82 and 39% after 48 hours, 15 day and 18 months DDT feeding. Alkaline phosphatase activity raised up to 180% at 3 day in ST-II experiment and 39% after 18 month treatment. The isocitrate dehydrogenase increased 85% and 22% during the same experiment. The hepatic cholesterol and free amino acids showed significant decrease in all experiments except later which remained unchanged in long term experiment. Glucose showed decrease (21%) in 48 hours and increased in 18 month feeding experiment. Soluble protein showed increase (33%) during 48 hours period, but decreased in 15 day (34%) and 18 month (20%) DDT feeding experiments. Total protein slightly decreased (21%) in 15 day and increased (21%) in 18 month treatment groups. Hepatic DNA showed increase which was maximum (42%) at 12 day duration while RNA showed significant decrease only during the long term experiment.

Key words: DDT, long term toxicity, short term toxicity, liver biochemistry, enzymes, metabolic alterations, Sprague Dawley rats.

INTRODUCTION

Dichlorodiphenyl trichloroethane (DDT) is one of the early chlorinated hydrocarbon insecticides used quite extensively throughout the world, including Pakistan. It has been used primarily for the control of insect pests of agriculture and medical importance. Like other organochlorine compounds it is very stable substance, which is only slowly degraded into different metabolites (Zaidi and Banerjee, 1987; Cicero *et al.*, 1992; Katayama and Matsumura, 1993; Thao *et al.*, 1993), which are also not less toxic than DDT itself. It's persistent nature (Hitch and

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Day, 1992; Longanathens *et al.*, 1993; Tanabe *et al.*, 1993; Hovinga *et al.*, 1993) and mobility in food chains is an important factor in chronic toxicity (Atuma, 1985a; Gartrel *et al.*, 1986; Kalra *et al.*, 1986). The deleteriouus effects of DDT on non-target organisms are largely due to its indiscriminate and unplanned use in domestic places, on fruits, vegetables, crops and in forests. Consequently the whole environment became polluted with DDT and its metabolites which are present in air, soil, rainwater, in large water bodies including aquatic life (Luco *et al.*, 1992; Hitch and Day, 1992; Tanabe *et al.*, 1993; Galassi *et al.*, 1994; Sukhoparova *et al.*, 1994).

After absorption into the system by inhalation, ingestion or contact with the skin, it is transported to liver through blood (Atuma, 1985b; Saxena *et al.*, 1987) where it becomes chemically linked with the lipid fraction especially with the lipoprotein complexes of the membrane system (Lee *et al.*, 1991; Antunes-Madieva, 1993; Teshke *et al.*, 1993). There are many reports about the storage of large amounts of DDT and its metabolite residues in animal tissues, such as liver, muscles, brain, adipose tissue and lactating organs (Rao, 1992; Hellou *et al.*, 1993; Kashyap *et al.*, 1993; Urdaneta, 1995).

Due to their persistence in the animal tissues, the DDT and its metabolites cause abnormal alterations in the physiology of the organism which is obviously a consequence of their interference with different metabolic pathways in the animal system (Sampson *et al.*, 1980; Ohyama *et al.*, 1982; Sanyal *et al.*, 1982; Dange, 1986). DDT and other organochlorine insecticides have qite a wide range of animal toxicity which is evident from the studies conducted by this and other laboratories (Ali *et al.*, 1988; Ali and Shakoori, 1988, 1990, 1993, 1994; Luco *et al.*, 1992; Hassoun *et al.*, 1993). Considerable information is availabe in literature on the toxicity (Kulshrestha *et al.*, 1986; Baronial and Sahai, 1993; Keith and Mitchell, 1993; Dickerson *et al.*, 1995) mortality, absorption and excretion of DDT and its metabolites in nontarget animals, especially in fish, birds and mammals (Hovinga *et al.*, 1993; Mason and Madsen, 1993; Somers *et al.*, 1993; Vourinen *et al.*, 1994). Since like other xenobiotics, DDT is metabolized in the liver, it is therefore likely to induce various amounts of toxicity in this organ.

The aim of the present study ws to determine the extent of biochemical toxicity induced by strong and weak doses of DDT for various durations in the liver of rat.

MATERIALS AND METHODS

Animals and their maintenance

Three groups of healthy Sprague Dawley rats were administered with various doses of DDT along with the feed for different durations. Two groups out of these with average weight 164.01 $\pm$ 9.73 g and about 5 month of age were used for two short term ST-I and ST-II experiments while one group with 106.37 $\pm$ 10.74 g and about 3 months of age was used for long term (LT) experiment. The animals were caged in 2.5 cubic feet iron cages and provided with food and water *ad libitum*. The rat feed was formulated in the laboratory by mixing, poultry feed (5 kg); fish meal (1 kg), wheat

flour (2 kg), molasses (100 g), and water (3 lit approx.).

Administration of pesticides

An organochlorine insecticide, dichloro-diphenyl trichloroethane (DDT; 1, 1,-trichloro-2, 2-*bis* (4-chlorophenyl ethane) was used as 75% powder and administered to animals orally along with the feed. Keeping in view the LD_{50} values of DDT one strong but sublethal dose (100 mg/kg B.Wt./day) was used for ST-I experiment. A week dose of 20 mg/kg B.Wt./day was used for ST-II and another weak dose (10 mg/kg B.Wt./day) was used for LT experiment.

For preparation of insecticide mixed diet for strong dose (ST-I) experiment, 800 mg of 75% DDT powder was mixed/kg of dry feed. In second ST experiment 525 mg of DDT was mixed with 3 kg of feed. For LT experiment 87.5 mg of toxicant was added/kg of rat feed. Since each rat on the average consumed about 30 g of feed daily, the calculated dose for ST-I experiment was 100 mg, for ST-II, 20 mg and for LT, it was 10 mg/kg B.Wt./day.

Procedure adopted

DDT toxicity to rat was studied as three different experiments, *i.e.*, two short term and one long term experiment which are mentioned below separately:

Short term experiment

In ST experiment, 2 groups with 4 animals each, were administered with DDT @ 100 mg/kg B.Wt./day for the total duration of 48 hours. Sampling was performed after every 24 hours in this experiment. In ST-II experiment, another group of 20 rats were administered with DDT @ 20 mg/kg body weight/day, for a total duration of 15 days. Samples for different analyses were collected after stipulated durations of 3, 6, 9, 12 and 15 days. Two groups of rats with 8 and 6 animals in each were used as control for two ST experiments, respectively.

Long term experiment

For long term experiment, a group of 12 animals was fed regularly on DDT mixed diet @ 10 mg/kg body weight/day for total period of 18 months. Every six month a group of 3 treated and 4-6 control animals were weighed anaesthetized and slaughtered, liver samples were collected and processed for various analyses.

Liver processing for biochemical analysis

Whole liver was taken out, weighed and then processed for biochemical studies. Liver weight, along with body weight was used to determine the relative liver weight (RLW; liver weight/body weight x 100).

Saline extract was prepared by homogenizing a piece of liver in ice cold isotonic

saline in a motor driven glass homogenizer. The homogenate was centrifuged at 8500 *g* in refrigerated centrifuge at 5°C to obtain clear supernatant, which was then used for different biochemical studies. For total proteins liver was completely dissolved in 2.5 ml of 0.5N NaOH and clear solution was used for total protein estimation. Another portion of liver was weighed and processed for the estimation of nucleic acids (DNA and RNA) content. For cholesterol estimation, ethanol extract was prepared.

Saline liver extract was used for estimation of alkaline phosphatase (AP; orthophosphoric monoester phosphohydrolase, alkaline optimum; EC.3.1.3.1) activity according to Kind and King (1954), isocitrate dehydrogenase (ICDH; threo-Ds-isocitrate: $NADP^+$ oxidoreductase; EC.3.7.3.2) activity according to Bell and Baron (1960), lactate dehydrogenase (LDH; NAD^+ oxidoreductase; EC.1.1.1.27) activity according to Cabaud and Wroblewski (1958), glutamate oxaloacetate transaminase (GOT; L-aspartate 2-oxoglutarate aminotransferase; EC.2.6.1.1 and glutamate pyruvate transaminase (GPT; L-alanine 2-oxoglutarate aminotransferase; EC.2.6.1.2) activities according to Reitman and Frankel (1957).

In addition some other biochemical contents *i.e.*, cholesterol according to Liebermann and Burchard reaction as described in Henry and Henry (1974), free amino acids (FAA) according to Moore and Stein (1954), glucose according to Hartel *et al.* (1969), protein according to Lowry *et al.* (1951) and nucleic acids (DNA-RNA) contents, were extracted according to Shakoori and Ahmad (1973) and estimated by Schmidt and Thannhauser procedure as described by Schneider (1957).

RESULTS

Body and liver weight

Tables I-III showed the changes in body weight gain and RLW after DDT administration for three different durations and dose levels *i.e.*, 48 hours (100 mg/kg/day), 15 days (20 mg/kg/day) and 18 months (10 mg/kg/day).

In 48 hours DDT feeding study, the body weight gain/day remained unaltered. However, RLW changed significantly (n=4) with 8.5% increase at 24 hour and 16.3% at 48 hours (Table I).

Uninterrupted DDT feeding for total period of 15 days did not produce any significant deviation in body weight gain and RLW of rats up to day 9. The percent weight gain/day, however showed 36%, 44% and 49% significant decrease at 9th, 12th and 15th day of DDT treatment. Similarly RLW showed regular increase from the day 6 onward which was 3.36, 3.36, 3.08 and 3.68 percent (n=9) on 6, 9, 12 and 15 day treatments (Table II).

In long term treatment although body weight gain was reduced after 6 and 12 months DDT feeding but significant reduction (20%) was found only at 18 month treatment. The change in RLW was significant in all treatments and showed 17%, 19%

and 29% increase (Table III).

Table I: Effect of feeding DDT-mixed diet (100 mg/kg body weight/day for 48 hours on the body and liver weight of albino rats

Parameters	Control (n=4)	DDT-fed	
		24 hours (n=4)	48 hours (n=4)
Percent weight gain per day	0.539±0.099[a]	0.563±110	0.487±0.089
Relative liver weight	2.59±0.042	2.80±0.059*	3.00±0.10*

[a]Mean ± SEM; Student's 't' test; *P<0.05

Table II: Effect of feeding DDT-mixed diet (20 mg/kg body weight/day) for 15 days on the body and liver weight of albino rats

Treatment	Percent weight gain/day	Relative liver weight
Control (n=6)	0.39±0.03[a]	2.76±0.05
3 days (n=3)	0.39±0.02	3.33±0.15
6 days (n=3)	0.35±0.03	3.36±0.05***
9 days (n=3)	0.25±0.04*	3.36±0.05***
12 days (n=3)	0.22±0.02**	3.08±0.08**
15 days (n=3)	0.20±0.02***	3.68±0.09***

[a]Mean±SEM, Student's 't' test; *P<0.05; **P<0.01; ***P<0.001.

Effect of various doses of DDT on liver enzyme activities are being mentioned in Tables IV-VII, which exhibited significant rise. The hepatic GOT activity showed 37 and 77% increase, while GPT showed 171% and 35% rise after 24 and 48 hours of DDT feeding. The LDH activity showed 61% and 71% increase during the same period. The AP and ICDH activities were not affected (Tables IV and VII). In second short term experiment (20 mg/kg/day) the AP activity was raised 180% after 3 days of DDT feeding, but normalized in the remaining experimental period. DDT administration produced mild alteration in hepatic transaminase activities. The GPT activity was reduced 25% after 3 days of feeding, while GOT activity was raised 74% and 61% after 12 and 15 days of DDT feeding. The ICDH and LDH activities appeared to be highly

sensitive to DDT feeding. The range of increase in ICDH activity was from 73% to 130% during 15 days of insecticide administration. The increase was more steady in LDH activity which was increased 29%, 41%, 62%, 60% and 82% after 3, 6, 9, 12 and 15 days of feeding (Tables V and VII). In 18 month DDT feeding (10 mg/kg/day) experiment the AP and LDH activities showed very prominent effect. The Ap activity was increased 64, 24 and 39% after 6, 12 and 18 months of feeding, respectively. The rise in LDH activity, was 42, 39 and 39% respectively during the same period. The hepatic GOT and ICDH activities remained unaltered except for 12 month group in which the former activity was raised by 2.12 fold, while the later enzyme showed 22% increase. The GPT activity was raised 1.24 fold, 5.27 fold and 1.86 fold after 6, 12 and 18 months of feeding (Tables VI and VII).

Table III: Effect of feeding DDT mixed diet (10 mg/kg body weight/day) for a period of 6-18 months on the body and liver weight of albino rats.

Parameters	6 months experiment		12 months experiment		18 months experiment	
	Control (n=6)	DDT-fed (n=3)	Control (n=4)	DDT-fed (n=3)	Control (n=6)	DDT-fed (n=3)
Percent weight gain per day	0.675[a] ±0.053	0.588 ±0.080	0.593 ±0.050	0.486 ±0.032	0.512 ±0.021	0.410** ±0.012
Relative liver weight	2.55 ±0.05	2.97* ±0.11	2.64 ±0.07	3.14** ±0.06	2.37 ±0.08	3.06** ±0.11

[a]Mean±SEM. Student's 't' test; *P<0.05; **P<0.01; ***P<0.001.

Biochemical analysis of liver

Several other hepatic biochemical components were tested for DDT toxicity (Tables IV-VII). The nucleic acid and total protein contents were not altered, while the soluble protein content increased 33% after 48 hours of DDT feeding. The FAA content showed drastic decrease (51% and 43% respectively) after 24 and 48 hours of DDT feeding. The glucose content also decreased (21%) after 48 hours of feeding. The cholesterol remained unaffected after 24 hours of feeding, but decreased by 44% on extending the DDT feeding for 48 hours (Tables IV and VII). In second short term experiment the cholesterol content decreased drastically *i.e.*, 68% after 3 days of DDT feeding. The decrease on day 6, 9, 12 and 15 was 43%, 54%, 40% and 38%, respectively. Total protein contents were not altered until day 12, when 16% decrease was recorded. After 15 days of feeding, the total proteins were found to decrease by 21%. The soluble protein, and FAA contents exhibited a continuous and constant decrease during 15 days DDT administration. The range of decline in soluble protein was from 24% to 35% while it was 31% to 54% in case of FAA content. The glucose content remained unaffected after 3 days of feeding while significant change with 40%, 75%, 99% and 88% increase was noted from 6 to 15 days of feeding (Table V). The rat liver showed several changes following DDT feeding as a mild dose for 18 months. The hepatic

soluble proteins were reduced 20% after 18 months of DDT feeding. The total protein content of liver decreased 33% after 6 months of DDT feeding, while the increase was 21% when DDT feeding was extended till 18 months. The glucose and RNA contents remained significantly low throughout 18 months DDT feeding study. The decrease was 59%, 39% and 31% in case of glucose while it was 45%, 26% and 39% in case of RNA. The DNA content was not affected until 18 months when it showed slight (13%) decrease. The FAA content remained unchanged during the study (Table VI).

Table IV: Effect of feeding DDT mixed diet (100 mg/kg body weight/day) for 48 hours on various biochemical components of albino rats liver

Parameters[b]	Control (n=5)	DDT-fed	
		24 hours (n=4)	48 hours (n=4)
AP (KAU/g)	0.80±0.16[a]	0.95±0.06	0.74±0.09
GOT (IU/g)	7.11±0.38	9.76±0.33**	12.61±1.79*
GPT (IU/g)	7.32±0.68	19.87±3.52**	9.91±0.86*
ICDH ($X10^3$ SU/g)	31.39±0.78	30.59±4.24	32.60±3.06
LDH ($X10^4$ IU/g)	56.56±4.43	90.96±4.94**	96.94±2.30**
Cholesterol (mg/g)	7.62±0.22	7.64±0.78	4.23±0.68**
FAA (μg/g)	399.21±18.13	194.85±17.51***	226.11±16.43***
Glucose (mg/g)	20.14±0.53	16.06±1.41*	16.00±1.88*
Soluble protein (mg/g)	111.18±5.08	122.06±8.06	158.45±8.45***
Total protein (mg/g)	199.33±6.11	211.58±16.29	218.44±11.58
DNA (mg/g)	3.84±0.44	2.64±0.50	3.68±0.58
RNA (mg/g)	9.53±0.55	7.86±0.90	9.61±0.29

[a]Mean±SEM, Student's 't' test; *P<0.05; **P<0.01; ***P<0.001.

[b]Abbreviations used: AP, alkaline phosphatase; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; ICDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; FAA, free amino acids; KAU, (King Armstrong Unit), liberation of 1 mg of phenol in 15 minutes under the test conditions; IU (International Unit), transformation of 1 micromole of sbstrate/ minute under the test conditions; SU (Sigma Unit), amount of enzyme that will produce 1 nanomole of NADPH in 1 hour under the test conditions.

Table V: Effect of feeding DDT mixed diet (20 mg/kg body weight/day) for 15 days on various biochemical components of albino rat liver.

Parameters	Control (n=5)	DDT-fed 3 days (n=4)	6 days (n=4)	9 days (n=4)	12 days (n=4)	15 days (n=4)
AP (KAU/g)	0.64[a]	1.79***	0.87	0.61	0.62	0.43
	0.02	0.21	0.11	0.05	0.06	0.12
GOT (IU/g)	7.34	8.79	8.89	11.03	12.80*	11.82**
	0.17	2.26	1.28	1.77	1.51	1.15
GPT (IU/g)	11.07	8.29*	12.10	12.86	10.45	10.31
	0.25	0.83	0.59	1.38	2.25	1.29
ICDH ($X10^3$ Sigma U/g)	20.10	46.32**	36.71*	36.54*	36.90**	37.25*
	3.50	6.60	5.88	4.44	3.09	5.10
LDH ($X10^4$ IU/g)	51.90	67.14**	73.12**	84.27**	83.13**	94.61***
	1.20	3.60	5.48	9.11	8.84	5.15
Cholesterol (mg/g)	15.72	5.06***	8.91**	7.26***	9.47***	9.77**
	0.35	0.95	1.42	0.71	0.37	1.18
Free amino acids (μg/g)	319.95	147.09***	163.46***	192.73***	222.23**	220.75**
	12.68	5.15	7.89	12.36	23.41	18.97
Glucose (mg/g)	12.93	10.56	18.11*	22.61***	25.75**	24.28**
	0.61	1.78	1.38	1.65	2.31	2.66
Soluble proteins (mg/g)	152.99	116.28**	99.44**	106.45*	104.25**	100.88**
	5.62	7.17	4.24	11.45	9.58	7.99
Total proteins (mg/g)	237.67	224.83	211.02	204.59	198.49**	188.35**
	7.45	12.15	12.73	11.80	9.37	10.81
DNA (mg/g)	2.42	2.70	3.19*	3.40*	3.43*	3.38*
	0.17	0.21	0.24	0.25	0.33	0.25
RNA (mg/g)	8.62	8.92	9.91	9.26	8.83	7.82
	0.38	0.73	0.94	0.77	0.78	0.62

[a]Mean $\pm$ SEM. Student's 't' test; *P<0.05; **P<0.01; ***P<0.001.
For other details, see Table IV.

Table VI: Effect of feeding DDT mixed diet (10 mg/kg body weight/day) for 6-18 months on the activities of various hepatic enzymes of albino rats.

Parameters	6 months		12 months		18 months	
	Control (n=6)	DDT-fed (n=3)	Control (n=4)	DDT-fed (n=3)	Control (n=6)	DDT-fed (n=3)
AP (KAU/g)	0.74[a] ±0.05	1.22*** ±0.08	1.12 ±0.07	1.39* ±0.04	1.08 ±0.08	1.49** ±0.11
GOT (IU/g)	8.66 ±0.54	6.09 ±0.18	7.56 ±0.46	16.05*** ±0.49	6.79 ±0.23	6.60 ±1.13
GPT (IU/g)	7.71 ±0.68	9.55 ±0.53	5.02 ±0.54	26.47*** ±3.03	6.05 ±0.60	11.26 ±0.62
ICDH ($X10^3$ Sigma U/g)	33.29 ±6.08	29.12 ±1.44	49.49 ±2.52	60.33* ±1.66	42.50 ±5.70	45.11 ±3.56
LDH ($X10^4$ IU/g)	58.29 ±2.99	82.64* ±7.56	54.25 ±5.37	75.17* ±0.89	44.60 ±0.30	61.81 ±5.33
Cholesterol (mg/g)	5.97 ±0.29	6.89 ±0.76	9.01 ±0.32	6.32* ±0.70	10.50 ±1.21	7.11* ±0.39
Free amino acids (μg/g)	217.29 ±7.70	221.83 ±21.81	197.98 ±14.18	216.00 ±13.41	170.12 ±11.59	187.47 ±13.04
Glucose (mg/g)	30.87 ±2.32	12.71*** ±1.22	37.12 ±1.37	22.75** ±3.21	38.32 ±3.38	26.11* ±3.23
Soluble proteins (mg/g)	124.92 ±4.67	106.73 ±10.96	135.22 ±9.96	111.48 ±17.23	159.00 ±2.58	128.08* ±10.93
Total proteins (mg/g)	216.96 ±13.05	145.88 ±15.76	208.48 ±6.70	228.54 ±12.38	227.60 ±11.88	274.41* ±0.65
DNA (mg/g)	3.05 ±0.11	3.15 ±0.16	2.54 ±0.14	2.78 ±0.30	3.06 ±0.12	2.66* ±0.09
RNA (mg/g)	9.68 ±0.62	5.35* ±0.86	10.39 ±0.31	7.68*** ±1.09	11.30 ±0.49	6.86*** ±0.26

[a]Mean ± SEM, Student's 't' test; *$P<0.05$; **$P<0.01$; ***$P<0.001$. For other details, see Table IV.

Table VII: **Percent increase (+) or decrease (-) in varios biochemical components of rat liver fed on DDT mixed diet for various durations.**

Parameters	Duration of treatment									
	24 h	48 h	3 days	6 days	9 days	12 days	15 days	6 months	12 months	18 months
AP	+19	-8	+180***	+36	-54	-3	+33	+64***	+24*	+39***
GOT	+37**	+77*	+20	+20	+50	+74**	+61**	-42	+112***	-3
GPT	+171**	+35*	-25*	+9	+16	-6	+25	+19	+527***	+86***
ICDH	-3	+11	+130*	+73*	+83*	+84**	+85*	-13	+22*	+6
LDH	+61**	+71**	-29**	+41**	+62**	+60**	+82***	+42*	+39*	+39*
Cholesterol	+1	-44**	-68**	-54**	-43***	-40***	-38**	+15	-18*	-32*
Glucose	-20*	-21*	-18	+40	+75	+99	+88	-59***	-39**	-32*
FAA	-51***	-43***	-54***	-40***	-49***	-31**	-31**	+2	+9	+10
Soluble protein	+10	+33**	-24**	-31**	-35*	-32*	-34**	-14	-18	-20*
Total protein	+21	+10	-5	-11	-14	-16*	-21**	-33*	-10	+21*
DNA	-31	-4	+12	+40*	+32*	+42*	+40*	+3	+9	-13*
RNA	-18	+1	+4	+15	+7	+2	-9	-45**	-26***	-39***

*P<0.05; **P<0.01; ***P<0.001 (Student's "t" test).
For abbreviations, see Table IV.

DISCUSSION

Body and liver weight

DDT treatment to rats @ 100 mg/kg/day for 48 hours did not produce any change in body weight gain, however, at 20 mg dose level the gain in body weight was reduced significantly at day 9, 12 and 15. In mild dose (10 mg/kg/day) *i.e.*, long term experiment, the inhibition was observed at 18 months of continuous feeding of DDT-mixed feed. The RLW in all treatments showed very consistent increase irrespective of the dose and duration of treatment. Several workers have conducted studies regarding changes in growth rate of animals after administration of different xenobiotics including DDT (Kohli and Venkitasubramanian, 1975; Sampson *et al.*, 1980; Laborda and De La Pena, 1983). Similar results have also been reported by Darsie *et al.* (1976). Kimbrough *et al.* (1971) showed that rats gained less weight after giving 500 ppm DDT for 6 weeks. Laborda and De La Pena (1983) co-related the decrease in body weight gain with dose and time of DDT treatment. On the other hand Cabral *et al.* (1982) did not report any adverse effects of DDT on body growth up to 500 ppm dose. Gain in body weight is a typical response of liver against variety of toxicants (Stevenson and Walker, 1969; Wright *et al.*, 1972; Walker *et al.*, 1973). Liver enlargement was detected in mice after 10 ppm dieldrin, 400 ppm lindane and 100 ppm DDT at 68 weeks of treatment. This increase in liver weight may be due to the increased accumulation of triglycerides in the liver (Kohli *et al.*, 1975) or it may be due to increase in smooth endoplasmic reticulum demonstrated with the electron microscope (Kimbrough *et al.*, 1971) which in turn corresponds to increase in microsomal drug metabolizing enzyme activity of the liver (Remmer and Marker, 1963; Chhabra and Fouts, 1973; Down and Chasseaud, 1981; Satya Narayan *et al.*, 1985). Liver microsomal proteins are also ascribed to increase after DDT feeding (Sanchez, 1967; Satya Narayan *et al.*, 1985) which may also be responsible for increase in liver weight.

Liver biochemistry

The various hepatic enzymes behaved uniformly following treatment of rats with various doses of DDT. In 48 hours DDT feeding (100 mg/kg/day) experiments, the hepatic AP and ICDH activities remained unchanged, while GOT, GPT and LDH increase considerably. In 15 day DDT feeding experiment, only hepatic LDH and ICDH activities showed consistent increase while AP, GOT and GPT activities occasionally increased. Some consistent and significant changes were however, observed in the various hepatic enzymes tested at 6-18 months of DDT feeding @ 10 mg/kg body weight/day. The GOT and GPT activities showed prominent deviation from the control.

DDT has already been reported to induce hepatic enzymes (Chadwick *et al.*, 1975). The increase in activities of various hepatic enzymes may be due to induction of these enzymes after DDT treatment. Suhasini *et al.* (1979) have also reported increase in hepatic GOT and GPT activities in frog after DDT administration. Agrawal *et al.* (1978) have shown increased succinic dehydrogenase and transaminase activities in brain, liver, kidney, adrenal and spleen of rhesus monkey after single dose of DDT as 150 mg/kg body weight. Amylase, LDH, Mg^{++} activated ATPase and AcP increase in some of these tissues, while AP activity decreased in all organs except kidney (Agrawal *et al.*,

1978). Ramalingam (1985) has reported decrease in hepatic and muscle LDH activity. The isozyme pattern also indicated marked variations from the control values which, according to author may be due to the alteration in oxidative capacity of these tissues.

The liver tissue reacts to DDT feeding by an increase in large number of biochemical components. The activities of AP, LDH, GOT and GPT enzymes and sugar content of hepatic tissue increased significantly after milder doses of DDT. Stronger doses of DDT do not behave differently. The raised level of different enzymatic activities of liver could be because of (*i*) liver damage followed by liver proliferation or (*ii*) because of increase in the synthesis of these particular proteins, which has been stimulated by DDT treatment (Cappon and Nicholls, 1973, 1975).

The hepatic AP activity increased significantly following 18 months of daily DDT feeding which may indicate hepatobiliary disease or some kind of bone abnormality. Saigol *et al.* (1982) has shown significant inhibition of hepatic and renal AcP and AP following one hour feeding of single dose of (60mg/kg body weight) DDT. Haowever, Shaffi (1982) reported elevated hepatic and renal AcP and AP in 3 species of fish exposed to sublethal doses of DDT. The raised transaminase (GOT, GPT) and LDH activities most probably reflected enhanced amino acid catabolism and gluconeogenesis. Elevated GOT and GPT activities were also shown by Borady *et al.* (1983) in rats fed DDT. This was also confirmed by decrease in glucose in 48 hours and 18 months and FAA in 48 hours DDT feeding experiments in liver. The stimulation of gluconeogenesis was reported in isolated hepatocytes from rats fed 1000 ppm DDT for 2 weeks (Story and Freedland, 1979).

In 15 days DDT feeding study the hepatic glucose concentration was not only recovered but showed increase which may restrict or partially inhibit the gluconeogenesis with partial depression of liver transaminase activities. The rise in glucose may also be due to inhibition of insulin by DDT (Malaisse *et al.*, 1971; Yau and Mennear, 1977). Kacew *et al.* (1972) and Kacew and Singhal (1973) reported that DDT administration enhanced the key gluconeogenesis enzymes in rat liver and kidney. The increased ICDH activity in 15 day and 12 month DDT feeding indicated increased energy generation by increasing the oxidation or catabolism of amino acids and sugars. There are several reports about the inhibition of hepatic LDH activity after DDT feeding in animals and also *in vitro* studies (Hendrickson and Bowden, 1976; Meany and Pocker, 1979). The present findings are in contrast to these studies. The increase in soluble proteins at 48 hours DDT feeding was an indication of slight injury to liver parenchyma cells or it may be due to the incorporation of amino acids into the proteins. The sugar metabolism was enhanced which is required for various biochemical processes inside the cell (Haynes, 1972; Kacew *et al.*, 1972). The cholesterol metabolism, however, appears to be hindered or inhibited, partly or wholly.

Besides enzymes, liver FAA contents seem to be the most prominent and sensitive parameters. The hepatic FAA, glucose and cholesterol content decreased 43 and 44% while soluble protein showed 33% increase only after 48 hours of DDT feeding. In 15 day feeding experiment, however, all hepatic biochemical components, except glucose, decreased significantly. The cholesterol, FAA, total proteins and soluble proteins showed decrease of 38%, 31%, 21% and 34%, respectively following 15 day DDT

feeding. Increased serum protein in our results after abnormal liver function may responsible for decreased hepatic soluble proteins in 15 day and 18 month DDT administration, while total proteins may be decreased due to decrease in body weight of rats as evident in our results. No consistent pattern of total proteins was visible in 18 months long DDT feeding experiment. After 18 months of feeding as mild dose the hepatic glucose and soluble proteins showed 32% and 20% decrease respectively, while total protein showed 21% increase.

The nucleic acid contents of liver showed some variable changes in short and long term DDT feeding experiments. In 48 hour feeding experiment, both DNA and RNA remained unchanged, while in ST-II experiment, the RNA content remained unchanged and DNA content increased 40% after 15 days of DDT feeding. In long term feeding experiments the DNA content decreased 13% after 18 months of feeding, while RNA content decreased 39% during the same period. The fall in soluble and total proteins also possible due to decrease in protein synthesis as confirmed by decrease in RNA contents. The decline in DNA content at 18 months DDT feeding was probably due to necrotic or degenerative changes in hepatic cells, while increased DNA in short term feeding (15 day) could be due to cell proliferation which may be the function of estrogen like effect of DDT as many workers reported this property of DDT (Gellert *et al.*, 1972; Balazs, 1979). Ireland *et al.* (1980) showed increased DNA synthesis by DDT in uterine tissue but this synthesis was 20-40% less than 17 beta-estradiol which is a steroid hormone.

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VARIATIONS OF ABO AND Rh BLOOD GROUP ALLELE FREQUENCIES IN THE POPULATION OF SIND (PAKISTAN)

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Abstract.- Percentage distribution and allele frequencies of ABO and Rh-phenotypes are described in the cities of the Sind province, Pakistan. Sex differences and relationship between the distribution of ABO and Rh-phenotypes are discussed. A significant differences in the distribution of ABO-phenotypes in the males and females are seen in Jacobabad, Mirpur Khas, Nawabshah, Sukkur and Thatta. Average allele frequencies for ABO and Rh groups in the population of Sind are A=0.17; B=0.22; O=0.61; d=0.15.

Key words: Blood group, allele frequency.

INTRODUCTON

The genotype and phenotype frequencies for ABO and Rh blood groups have been investigated by different authors from various parts of the world and found that the incidence of blood groups varies from one race to another. In European population the frequency of allele A is higher than the frequency of allele B as described in Swedish (Beckman, 1972), Lipari-Italian (Warwick *et al.*, 1972) and Byellorussian and Poles (Oreknova, 1975) populations. Same is reported in Antham (Chileotin) Indians (Alfered *et al.*, 1970). In the populations of north and west Africa the frequency of allele A is still higher than allele B as reported for Aswan and Nubians (Azim *et al.*, 1974), North sinai (Hamza *et al.*, 1976) and Nigerian (Odeigah, 1990) populations. In contrast in southeast Asian populations the frequency of allele B is noticeably greater than allele A as reported in Rajasthan (Hurkat *et al.*, 1971) and Punjab, Pakistan (Shami and Kamboh, 1979). The variation in ABO distribution envisages not only the frequency distribution of phenotypes and genotypes, but also studies were carried out to unveil other variables in relation to these blood groups. Such studies include differential fertility and mortality in relation to ABO groups (Bennt and Walker, 1955), effects of natural selection on ABO locus (Chung and Morton, 1961), ABO-incompatibility (Lauriksen *et al.*, 1975), association with goiter (Harison *et al.*, 1976), thyroid cancer (Hernandez *et al.*,1980), Breast cancer (Levine *et al.*, 1981), Cardiac infection (Pdatt *et al.*, 1985), carcinoma of stomach (Mecklin *et al.*, 1988), lung cancer (Cerny *et al.*, 1993), Linkage between ABO and nail patella (Renwick and Lawler, 1955) and Rh and marfan syndrome (Mace and Margaret, 1979).

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The present investigation shows the distribution of phenotypes and genotypes of ABO and Rh blood groups in the population of Sind (Pakistan) based on data from 13 cities.

MATERIALS AND METHODS

The data are based on records of Blood Transfusion Centers and Hospitals in different cities of Sind (Pakistan). The data were collected from Badin, Dadu, Hyderabad, Jacobabad, Karachi, Khairpur, Larkana, Mirpur Khas, Nawabshah, Sanghar, Shikarpur, Sukkur and Thatta (Fig. 1). The individuals tested for ABO system were also typed for Rh group i.e., D-positive and D-negative. Chi-square tests were carried out for different analysis.

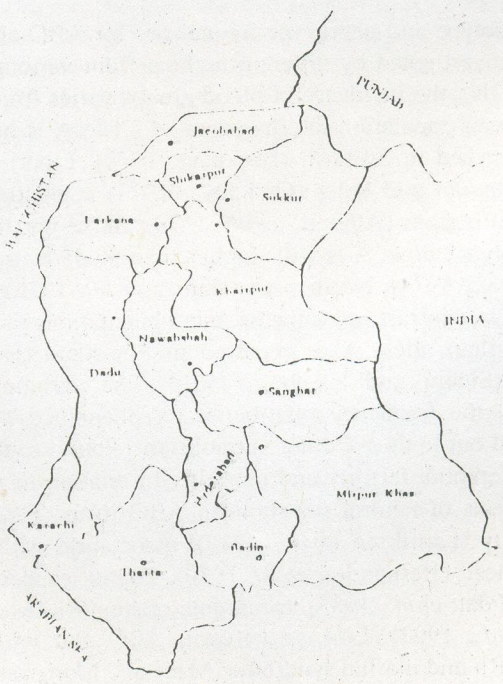

Fig. 1. Map of Sind (Pakistan) showing the cities of data collection.

RESULTS

The highest percentage for A-phenotype is seen in Shikarpur (28.68) followed by Thatta (27.84), Nawabshah (27.64), Mirpur Khas (27.56), Jacobabad (26.57) and Sanghar (26.43). A remarkably low percentage for A-phenotype is seen in Dadu (22.10), sukkur (21.97) and Larkana (18.62). Group B-phenotype shows highest percentage in Karachi (36.04) followed by Sukkur (35.59), Hyderabad (34.86) and Jacobabad (34.77). The lowest percentage values are seen in Dadu (28.15) and Shikarpur (23.23).

The highest percentage for AB-phenotype is observed in Karachi (9.36) followed by Mirpur Khas (8.64), Sanghar (8.41), Hyderabad (7.92) and Sukkur (7.62). The lowest percentage values are seen in Jacobabad (4.50), Thatta (4.10) and Larkana (3.90).

The O-phenotype shows higher percentage compared with A, B and AB-phenotypes. The highest percentage of O-phenotype is observed in Larkana (47.65) followed by Dadu (43.49) and Shikarpur (43.09). The lowest values are seen in Mirpur Khas (31.92) and Karachi (30.65).

The percentage distribution of ABO phenotypes in males and females is almost the same in different cities of Sind. Appreciable differences in A-phenotypes are seen in Mirpur Khas (♂♂ 30.69; ♀♀ 21.96), Thatta (♂♂ 26.56, ♀♀ 33.04) and Khairpur (♂♂ 27.64; ♀♀ 21.75). In B-phenotype differences are observed in Jacobabad (♂♂ 29.33; ♀♀ 40.37) and Mirpur Khas (♂♂ 29.27; ♀♀ 37.06). AB-phenotypes show difference in Mirpur Khas (♂♂ 5.83; ♀♀ 13.14), Badin (♂♂ 5.40; ♀♀ 8.97) and Sukkur (♂♂ 6.37; ♀♀ 9.43). In O-phenotype a difference ranging between 3.00-8.00 can be seen in most of the cities except prominent differences in Thatta (♂♂ 37.31; ♀♀ 26.25) and Sikarpur (♂♂ 47.17; ♀♀ 37.67).

Chi-square tests were carried out to find differences in the distribution of male and female ABO-phenotypes. Significant differences are observed in Jacobabad (X^2_3=15.35; $p<0.01$), Mirpur Khas (X^2_3=40.01; $p<0.001$), Nawabshah (X^2_3=15.15; $p<0.01$), Sukkur (X^2_3=10.24; $p<0.02$) and Thatta (X^2_3=11.83; $p<0.01$).

Chi-square tests were carried out to find goodness of fit to Hardy-Weinberg expectation in relation to ABO-phenotypes (Table I). The results are statistically non-significant showing agreement with Hardy-Weinberg expectation. Highly significant deviation from Hardy-Weinberg expectation is seen in Jacobabad (X^2_1=21.08; $p<0.001$), Nawabshah (X^2_1=14.73; $p<0.001$) and Thatta (X^2_1=26.24; $P<0.001$).

Data were also arranged to see distribution of ABO-phenotypes in combination with Rh-group. The different combination in which data were arranged are Rh^+/A, Rh^+/B, Rh^+/AB, Rh^+/O and Rh^-/A, Rh^-/B, Rh^-/AB, Rh^-/O.

Table I. **Allele frequencies and goodness of fit to Hardy-Weinberg expectation of ABO phenotypes in the cities of Sind (Pakistan)**

Locality	Blood Groups				Allele frequencies				
	A	B	AB	O	A	B	O	X^2_1	P
Badin	155	209	46	235	0.17	0.22	0.61	0.36	n.s
Dadu	307	391	87	604	0.15	0.19	0.66	0.92	n.s
Hyderabad	738	1070	243	1018	0.18	0.24	0.58	3.52	n.s
Jacobabad	266	348	45	342	0.17	0.22	0.61	21.08	<0.001
Karachi	1054	1586	412	1349	0.18	0.26	0.56	1.30	n.s
Khairpur	209	268	54	325	0.17	0.21	0.62	1.16	n.s
Larkana	234	375	49	599	0.12	0.19	0.69	1.47	n.s
Mirpur Khas	391	455	120	453	0.20	0.23	0.57	1.43	n.s
Nawabshah	681	795	162	826	0.19	0.22	0.59	14.73	<0.001
Sanghar	355	397	113	478	0.19	0.21	0.60	0.38	n.s
Shikarpur	195	149	34	293	0.19	0.15	0.66	1.05	n.s
Sukkur	331	537	115	526	0.16	0.25	0.59	0.46	n.s
Thatta	326	385	48	412	0.18	0.21	0.61	26.24	<0.001
Average					**0.17**	**0.22**	**0.61**		

n.s. = Non-significant

A higher percentage of Rh^+/A-phenotype is seen in Shikarpur (28.80), Nawabshah (27.90) and Thatta (27.91). A higher percentage of Rh^+/B-phenotype is seen in Karachi (36.26), Sukkur (35.56) and Hyderabad (35.07). Rh^+/AB-phenotype shows higher percentage in Karachi (9.31), Mirpur Khas (8.58), Sanghar (8.46) and Hyderabad (8.23). Rh^+/O-phenotype shows higher percentage in Larkana (47.74). Rh^-/A-phenotype shows higher percentage in Mirpur Khas (32.89) and Sukkur (27.54). A higher percentage of Rh^-/B-phenotype is seen in Badin (66.67) and Dadu (50.00). Rh^-/AB-phenotype shows higher percentage in Khairpur (100.00). Sanghar (71.42) and Shikarpur (66.67).

A higher percentage of Rh^+/ABO-phenotype is seen in males as compared to females in all cities except in Dadu (64.17), Khairpur (54.80) and Hyderabad (63.21) where females have higher percentage when all phenotypes are taken together. Rh^+/B (33.98) and Rh^+/AB (9.06) females from Badin, Rh^+/A (23.15) and Rh^+/AB (6.74) females from Dadu, Rh^+/AB females (8.62) from Hyderabad, Rh^+/B (40.33) females from Jacobabad, Rh^+/A (24.71) and Rh^+/B (10.42) females from Karachi, Rh^+/B (33.55) and Rh^+/AB (7.27) females from Khairpur, Rh^+/A (22.03), Rh^+/B (30.39) and Rh^+/AB (5.73) females from Larkana, Rh^+/B (37.58) and Rh^+/AB (12.53) females from Mirpur Khas, Rh^+/A (29.90) and Rh^+/AB (7.08) females from Nawabshah, Rh^+/A (31.21) and Rh^+/AB (10.83) females from Sanghar, Rh^+/A (24.14) and Rh^+/AB (9.25) females from Sukkur, Rh^+/A (33.19), Rh^+/B (5.67) females from Thatta show higher percentage for respective phenotypic combination compared with that of males.

Chi-square tests were carried out to see relationship of ABO-phenotypes in combination with Rh-positive and Rh-negative blood groups. Statistically significant results are obtained from Mirpur Khas (X^2_3=11.08; $p<0.02$), Nawabshah (X^2_3=10.83; $p<0.02$) and Thatta (X^2_3=24.02; $p<0.001$).

Chi-square tests were carried out to compare the distribution of ABO-positive phenotypes (A^+, B^+, AB^+, O^+) and ABO-negative phenotypes (A^-, B^-, AB^-, O^-) in males and females. Statistically significant results for ABO-positive phenotypes are observed in Jacobabad (X^2_3=15.58; $p<0.01$), Mirpur Khas (X^2_3=25.77; $p<0.01$), Nawabshah (X^2_3=9.02; $p<0.05$), Sukkur (X^2_3=9.75; $p<0.02$) and Thatta (X^2_3=11.01; $p<0.02$). Only two statistically significant results, regarding ABO-negative phenotypes, are obtained from Mirpur Khas (X^2_3=22.36; $p<0.001$) and Nawabshah (X^2_3=14.28; $p<0.01$).

Table II shows the allele frequencies for allele d in males and females, sexes combined in different cities of Sind. There are regional differences in the distribution of allele d. The highest frequency of allele d is seen in Mirpur Khas (0.32) and lowest in Thatta (0.03). In males and females the highest frequency for allele d is observed in Mirpur Khas (0.33 and 0.30 respectively). No case of Rh-negative males and females is reported from Thatta and Shikarpur respectively.

Table II. Frequencies of allele d in males, females and sexes combined in the cities of Sind (Pakistan).

Locality	Males	Females	Sexes combined
Badin	0.13	0.10	0.12
Dadu	0.04	0.03	0.04
Hyderabad	0.25	0.28	0.27
Jacobabad	0.11	0.12	0.11
Karachi	0.24	0.23	0.24
Khairpur	0.05	0.05	0.05
Larkana	0.12	0.11	0.12
Mirpur Khas	0.33	0.30	0.32
Nawabshah	0.31	0.22	0.27
Sanghar	0.06	0.11	0.07
Shikarpur	0.09	0.00	0.07
Sukkur	0.21	0.22	0.21
Thatta	0.00	0.07	0.03
Average	**0.15**	**0.14**	**0.15**

DISCUSSION

The present investigation shows differences in allele frequencies in different parts of the Sind (Pakistan). Lowest frequency of allele A is seen in Larkana and highest in Mirpur Khas. Allele B showed lowest frequency in Shikarpur and highest in Karachi. The lowest frequency of allele O is observed in Karachi and highest in Larkana. The variations in gene frequency may be due to random genetic drift (Wright, 1931; 1940 and Turaeva *et al*, 1984). The variations in gene frequency may be maintained due to balanced effects on natural selection (Ford, 1945). Thompson (1972) investigated that changes in population structure can produce large effect on gene frequencies in one generation as any normal selective force. At the moment it is not possible to show which of the three forces is more effective in the present population. Considering the marriage types, most of the marriages are between the relatives than between persons not known to be related. This would obviously encourage the incidence of certain alleles, but at the same time the respective phenotypes may show susceptibility to a certain disease which would have a selective effect on phenotypes.

The ABO-phenotypes show complete agreement to Hardy-Weinberg expectation except in Jacobabad, Nawabshah and Thatta (Table I). Shami and Kamboh (1979) showed highly significant deviation from Hardy-Weinberg expectation in four cities of the Punjab, Pakistan (Gujranwala, Multan, Sahiwal and Sialkot). The data show that the main cause of significant deviation from Hardy-Weinberg expectation is AB individuals, showing significant differences between observed and expected phenotypic values. This could be due to misclassification, inbreeding etc., (Beckman *et al.*, 1972). The probability of significant deviation from Hardy-Weinberg expectation due to misclassification seems to be low since discrepancy is seen only in AB individuals at three places. The possible explanation could be that selection is working against these phenotypes. Mourant (1974) suggest that certain blood group phenotypes are more susceptible to certain diseases resulting in loss of fertility. This would an important selective factor under certain conditions.

The data lack information regarding the association to certain disease with blood group, however, evidence derived from the existing data show that AB phenotype are more susceptible to smallpox (Vogal & Chakravartti, 1966), tuberculosis (Jain, 1970), chicken pox (Hepp *et al.*, 1975), cardiac infection (Pdatt *et al.*, 1985) and lung cancer (Cerny *et al.*, 1993).

The distribution of ABO-phenotypes in the two sexes show significant differences in five cities. Shami and Kamboh (1979) showed significant differences in the distribution of ABO-phenotypes in two sexes in the six cities of the Punjab (Pakistan).

The lowest frequency for allele d is seen in Thatta (0.03) and the highest in Mirpur Khas (0.32). The data do not show any record of Rh-negative male from Thatta and female.

from Shikarpur. Singh *et al.* (1974) showed absence of Rh-negative in Tibetans. No case of Rh-negative was recorded from Muzafargarh (Shami and Kamboh, 1979).

The population of Assam shows similarity with regard to ABO blood group frequencies with Karachi (Gupta and Sarthak, 1979). The populations are lying at the border of two countries. Although there is no mixing between two populations since the establishment of Pakistan, but still the two populations are maintaining more or less the same distribution of ABO blood group alleles. Shami and Kamboh (1979) showed resemblance of frequencies of alleles B and O in the population of Rajasthan (India) and Bahawalpur (Pakistan).

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BIRDS AS BIO-INDICATORS OF RADIOACTIVE NUCLIDES IN PAKISTAN

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Abstract: Pakistan is situated in South Asia and its Indus valley is the major wintering and residing ground for millions of waterfowl. The present study was aimed to analyze the presence of accidental (Chernobyl) and experimental air borne radioactivity through migratory and indigenous birds as they are the bio-indicators of environmental pollution. The study revealed that a higher concentration of radioactivity was traced in migratory birds coming from Northern Hemisphere, compared with local birds collected from vicinity of Dera Ghazi Khan (Pakistan). The food contents analyzed in bird's stomach showed that they utilized marshy as well as terrestrial vegetation. It is concluded that the ecosystems of Northern Hemisphere and Pakistan have been contaminated with radionuclides in the first, second and third trophic levels in our various ecosystem.

Key words: Birds as bio-indicators, radioactivity, Chernobyl environmental pollution.

INTRODUCTION

Radioactive contamination is increasing day by day due to peaceful uses of atomic energy, testing of nuclear weapons and accidental release from the nuclear power plants. The accidents which occur in the nuclear power plants are mainly responsible for sudden increase in the concentration of radionuclides in the environment. On such major accident happened on April 26, 1986 at Chernobyl Nuclear Power Plant in the former USSR. It was resulted in air borne release of mega-curie quantities of fission products which are now a part of the environment not only in the former Soviet Union but spread far beyond its boundaries (Hall, 1986).

The composition of fission products released from the accidents in nuclear reactor at Windscale in U.K. showed that in addition to the noble gases, the prominent nuclides were the volatile fission products such as iodine (I), tellurium (Te), cesium (Cs) and also smaller amounts of ruthenium (Ru), strontium (Sr), cerium (Ce) and barium (Ba), (Loutit *et al.*, 1960). In accidents, I is the dominant contaminant (British Medical Research Council, 1960). During vegetation most of the air borne radioactivity come in contact with the leaves and enter the food chain (Smith and Epstein, 1964). The above mentioned mode of entry is the main source of contamination by Ce whereas Sr is only

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absorbed by the leaf surface (Yamagata, 1963).

It is evident that the contamination of the ecosystem is continously increasing due to radioactive fallout. The accident at Chernobyl was of great magnitude than any other. There are continuous reports that both the aquatic and terrestrial ecosystems have been contaminated in the Northern Hemisphere.

According to some recent studies done in Norway radio-cesium from Chernobyl reactor has been detected in Eurasian woodcock and earthworms (Atle *et al.*, 1994). Pearce (1995) has also reported radiocesium in migratory birds in Northern Ireland, released during Chernobyl accident. Millions of migratory waterfowl breed in the Northern Hemisphere in which the majority of these breed in Russia and feed on various types of food available there. It is suspected that these birds might take up radioactivity through food. A large number of migratory waterfowl of Russia origin winter in the Indus valley of Pakistan. So far, it is not known whether these migratory birds contain radioactivity, if any, what is its impact on food chain through wetland and terrestrial ecosystem. Thus the study was undertaken to analyze the radioactivity in migratory and local waterfowl.

MATERIALS AND METHODS

Aquatic migratory birds were collected from vicinity of Dera Ghazi Khan district (D.G. Khan), Ghamagar lake and Bhila Hithar of Kasur district and Kandhkot of Jacobabad district. Starling and black winged Stilt as local wildfowl were also collected from D.G. Khan area. Specimens were kept at zero degree centigrade, continued to be enclosed in polythene bags.

Stomach contents of the birds were dried (Roberts and Dorothy, 1966) and weighed. Different stomach contents, seeds, leaf fragments, salt, snail shells and pebbles were separated from each other. Percentage ratio of each component to the total dry weight of stomach contents was calculated. Different leaf fragments and kinds of seeds were identified (Martin, 1961 and Core, 1959).

Radioactivity was detected in the migratory birds by means of solid state nuclear track detector (NTD), Trade Mark Cr-39. The NTD were placed in feathers, beaks, muscles and bones of the specimens. The contact was maintained by wrapping the cellophane tape around the sample and detector. Samples with detectors were kept in the freezer at 0°C for 15 days. The etching was done by placing exposed detectors of various sizes such as 2-5 cm in the etchant solution for 10 hours at 70°C. Number of alpha tracks formed on NTD were counted per square centimeter of the NTD surface, under the microscope. The exposed NTD-Cr, 39 were subjected to microphotography.

RESULTS AND DISCUSSION

Sturnum muscles of pochard (*Aythya ferina*) and coot (*Fulica atra*) showed 75 and

70 tracks of alpha particles per centimeter square of NTD, respectively. Mallard (*Anas platyrhynchos*) and gadwall (*Anas strepera*) showed 53 and 51 tracks of alpha particles per centimeter square of NTD, respectively. Teal (*Anas crecea*) and black winged stilt (*Himantopus himantopus*) showed 15 and 12 tracks of alpha particles per centimeter square of NTD, respectively. Pintail (*Anas acuta*) and shoveler (*Anas clypeata*) showed 32 and 30 tracks of alpha particles per centimeter square of NTD, respectively. Starling (*Sturnus vulgaris*) showed 16 tracks of alpha particles on one centimeter square of NTD (Fig.1).

These results revealed that pochard and coot showed maximum radioactivity in their body while teal and black-winged stilt showed minimum concentration of radioactive nuclides. At this stage a question arises as to why there is a variation in the number of tracks of alpha particles in various birds? The first reason is that the breeding and feeding areas of birds indicating elevated redioactivity were coming from the adjacent areas of the Chernohyl while other birds were residing away from high radioactivity zones. The second reason seems to be the food of pochard and coot which might consist of plants having high concentration of radionuclides. Their stomach contained seeds of *Melilotus alba* and *Trignoella* spp. and *Oryza sativa* (rice). There were also leaves and salts (Table 1) in composition with other food materials. It appears that pochard and coot had a similar feeding ground and had already picked up radionuclides from the parent ecosystems.

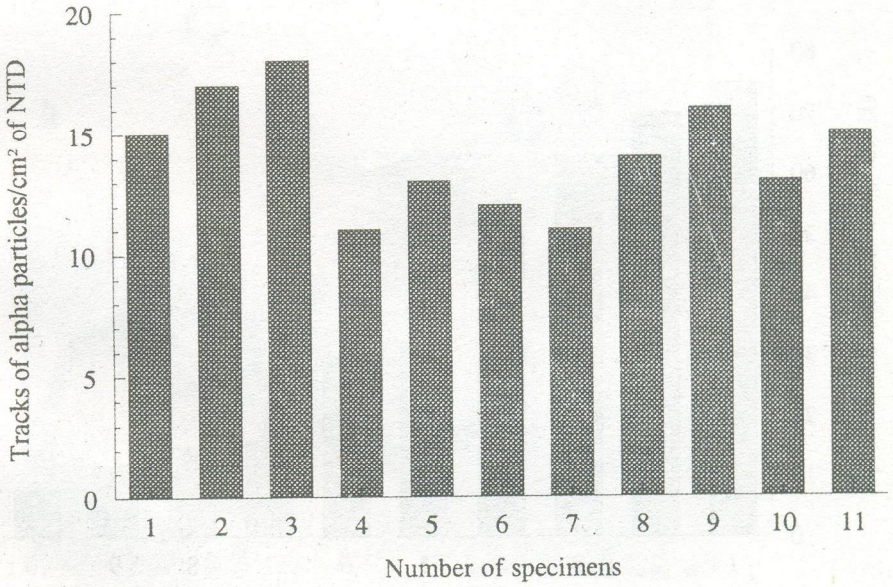

Fig. 1: Histogram showing radioactivity in different specimens of teal, *Anas crecca*.

Mallard and gadwall also showed the highest level of radioactivity with 53 and 51 tracks of alpha particles per centimeter square of NTD, respectively. These were collected from Dera Ghazi Khan district. Their stomach contents included 56.78% seeds and 12.92% leaves of *Melilotus alba* with 18.92% salts and 11.37% pebbles (Table 1). They might have obtained radionuclides from the parent ecosystem through contaminated food chain. Pintail and shoveler showed the third highest level of radioactivity with 32 and 30 tracks of alpha particles per centimeter square of NTD, respectively. The stomach contents of pintail included seeds (81.99%) with 78% rice (*Oryza sativa*). It appears that pintail has a feeding ground in semiaquatic areas like paddy fields. It might have fed on the similar type of vegetation in its breeding grounds and acquired radionuclides. Relative distribution of fall out activity in component parts of cereal crops *i.e.*, distribution of radioactivity in different parts of paddy by Sr crops are exposed to and collect as much fall out contamination as other vegetation. De-husked grain skinned food fruit is the least contaminated part of the plant and milling of rice was found to eliminate the more contaminated part of the grain (Ali, 1979). The same is true for shovelers which were collected from Dera Ghazi Khan and Kasur districts. They might have also received radionuclides from the contaminated vegetation of their breeding grounds. Teal showed minimum radioactivity with a range of 12-18 tracks of alpha particles per centimeter square of NTD (Fig.2). Their stomach contents showed seeds and leaf fragments of *Echinochloa* spp., *Amarants* spp., *Medicago denticulatum* and *Trigonella* spp. with insects, smashed shells of snails and salts. This analysis showed that, these birds might have obtained radionuclides by feeding on contaminated vegetation from their breeding sites.

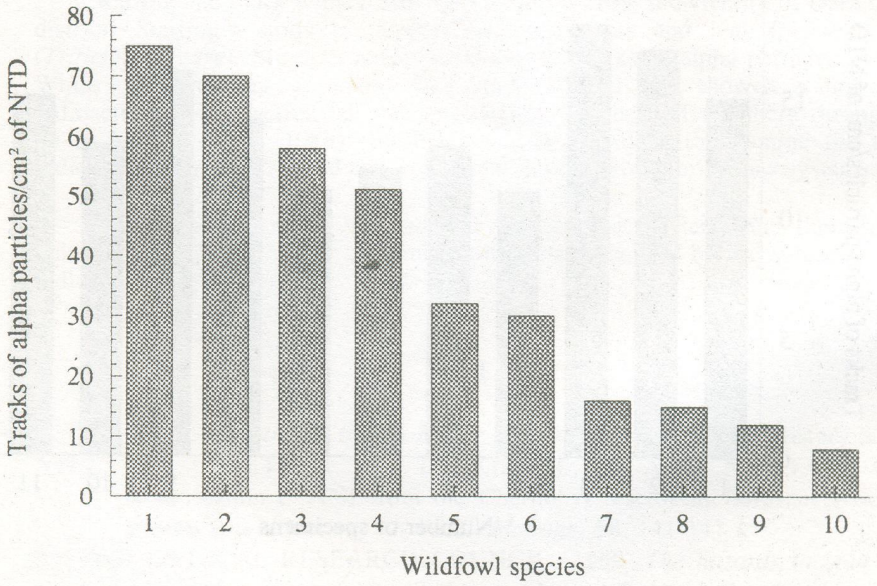

Fig. 2: Histogram showing radioactivity in different species of wildfowl. 1, pochard; 2, coot; 3, mallard; 4, gadwall; 5, pintail; 6, shoveler; 7, starling; 8, teal; 9, black winged stilt; 10, control.

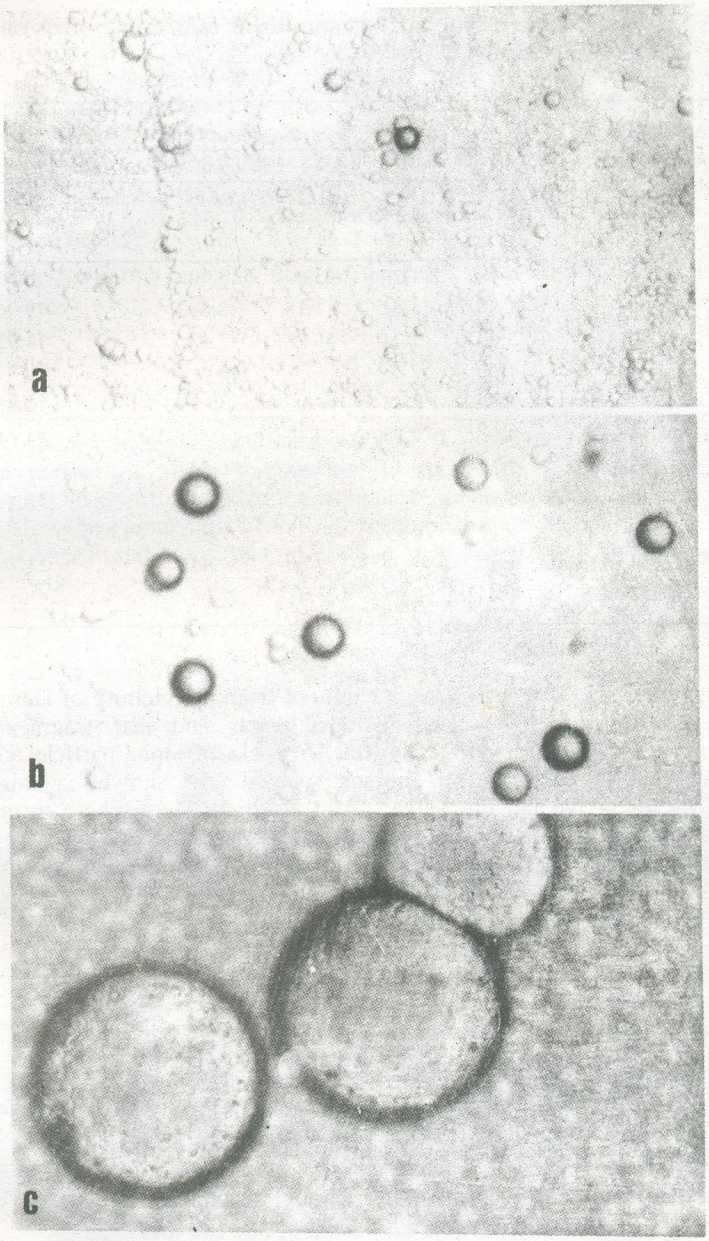

Fig. 3: Microphotograph of nuclear track detector (NTD) showing tracks of alpha particles; a, unexposed (100X); b, exposed (100X); c, exposed (400X).

Table 1: Stomach contents in various species of migratory birds collected from different localities.

Species	Area of collection	Food analysis						
		Weight (gm)		Food contents (%)				
		Wet	Dry	Seeds	Leaves	Snail shells	Salts	Pebbles
Pochard	Kandh Kot	3.90	1.29	65.00	15.50	-	19.50	-
Coot	D.G. Khan	11.80	5.52	1.16	18.88	-	79.96	-
Mallard	D.G. Khan	3.80	1.32	56.78	12.92	-	18.92	11.38
Gadwall	Kandh Kot	11.00	7.58	-	100	-	-	-
Pintail	Kasur	7.56	5.55	81.99	-	-	10.81	7.20
Shoveler	Kasur	4.01	2.75	7.27	-	-	74.54	18.19
Starling	D.G. Khan	2.52	0.46	43.48	56.52	-	-	-
Teal	D.G. Khan	2.98	1.36	44.52	-	-	55.48	-
	Kasur	1.19	0.57	40.35	-	59.65	-	-
	Jacobabad	2.36	1.01	19.80	56.44	-	23.76	-
B.W Stilt	D.G. Khan	1.31	0.30	23.34	36.66	-	-	40.00

Starling and black winged stilt were captured from the vicinity of Dera Ghazi Khan district. Starling's stomach contents included seeds and leaf fragments of wheat (*Triticum vulgare*). Sturnum muscles showed 16 tracks of alpha particles/cm^2 of NTD. While other resident waterfowl like black winged stilt showed a fair amount of radioactivity. Radioactive fall out spread from Northern Hemisphere through Pakistan up to Kuwait (Hull, 1986). Further research is needed to examine the quantity of radionuclides in the 1st, 2nd and 3rd trophic levels in our various ecosystems.

It is evident that the wetland and terrestrial ecosystems and their resources in Pakistan have also received contamination of radionuclides by the spread of Chernobyl radioactive fall out (Fig.3).

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COMPARATIVE STUDY OF SOME DENTITIONS OF *SELENOPORTAX VEXILLARIUS* PILGRIM AND *SELENOPORTAX LYDEKKERI* PILGRIM (ARTIODACTYLA, BOVIDAE, MAMMALIA)

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Abstract: Pilgrim (1937) based this genus upon a collection from the various localities of Pakistan and India. This collection comprised a skull lacking the maxilla and dentition and most of the basicranium (A.M.N.H. No. 19748), many isolated teeth and a left horn-cor (Brit. Mus. No. M. 24028). Pilgrim (1937) referred all these specimens to the genus *Selenoportax* Pilgrim and added one species in it, *S. vexillarius* Pilgrim. Lydekker (1884) described and figured seven different specimens under the name *Boselaphus* sp. from Punjab, no exact locality has been mentioned by him. These specimens were maxilla, mandible and isolated teeth. Pilgrim (1910) established the species *Boselaphus lydekkeri* on this material. However, Pilgrim later (1937) transferred all these specimens to the genus *Selenoportax* and species *S. lydekkeri*. Gentry (1974) proposed that referred teeth of a supposed species *S. lydekkeri* must be regarded as of uncertain identity. Recently, more material of the genus *Selenoportax* has been collected from the Siwaliks. This material consists of two fragmentary crania, mandible and many isolated teeth. These specimens are referred to the genus *Selenoportax* in three species, *S. vexillarius, S. lydekkeri* and a third new species *S. dhokpathanensis*. In general, the main difference seen between two species, *S. vexillarius* Pilgrim and *S. lydekkeri* lies in the size of upper teeth, M_3 and mandible. There is no difference in the structure of the teeth. The teeth referred to species *S. vexillarius* are comparatively smaller than those of *S. lydekkeri* and are somewhat less hypsodont and have comparatively weaker median basal pillars in the upper molars. *Selenoportax lydekkeri* which was considered doubtful by Gentry (1974) has been regarded as a valid species of the genus *Selenoportax* Pilgrim.

Key words: *Selenoportax, Boselaphus,* Siwaliks.

INTRODUCTION

The genus *Selenoportax* is known since 1937 when Pilgrim studied and described the Siwalik Bovidae in the American Museum of Natural History (Am. Mus.). He added one species *S. vexillarius* in it. Lydekker (1884) described and figured seven different specimens under the name *Boselaphus* sp. from the Siwaliks. These specimens were maxilla, mandible and isolated teeth. Pilgrim (1910) erected the species *Boselaphus lydekkeri* on these specimens. However, Pilgrim later (1937) transferred all these specimens to the genus *Selenoportax* and species *Selenoportax lydekkeri*. Recently, more material of *Selenoportax* has been collected from the Middle Siwaliks. This material comprises mandibles and many isolated upper and lower teeth, M_3 and mandible. These specimens are referred to species *S. vexillarius* and *S. lydekkeri*. The main difference seen between two species lies

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in the size of upper teeth, M_3 and mandible. There is no difference in the structure of the teeth. The species *Selenoportax lydekkeri* which was considered doubtful by Gentry (1974) has been regarded as a valid species of the genus *Selenoportax* Pilgrim. The classification is based on Simpson (1945).

Systematic Account

Order:	Artiodactyla Owen
Suborder:	Ruminantia Scopoli
Infraorder:	Pecora Linnaeus
Superfamily:	Bovoidea Simpson
Family:	Bovidae Gray
Subfamily:	Bovinae Gill
Tribe:	Boselaphini Simpson
Genus:	*Selenoportax* Pilgrim, 1937

Type species

Selenoportax vexillarius Pilgrim 1937.

Included species

i. *Selenoportax vexillarius* Pilgrim, 1937
ii. *Selenoportax lydekkeri* Pilgrim, 1937
iii. *Selenoportax dhokpathanensis* (new species)

Type

A skull lacking maxilla and dentition and most of the basicranium, Am. Mus. No. 19748.

Locality

Hasnot, district Jhelum, Punjab, Pakistan.

Horizon

The Nagri stage of the Middle Siwaliks.

Diagnosis

Cheek teeth large, strongly hypsodont, enamel very rugose, upper molar quadrate, with strong and divergent styles, median ribs well developed, strongly developed medial basal pillars on upper and of moderate size on the lower molars.

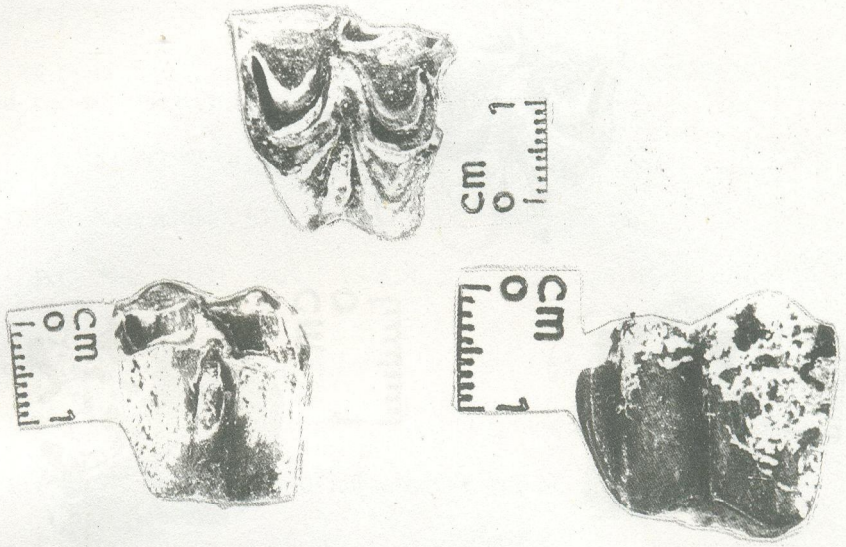

Fig. 1: *Selenoportax vexillarius*, an isolated right M^2 (P.U.P.C. No. 85/9) from Hasnot, district Jhelum, Punjab, Pakistan. A, Crown view; B, Inner view; C, Outer view.

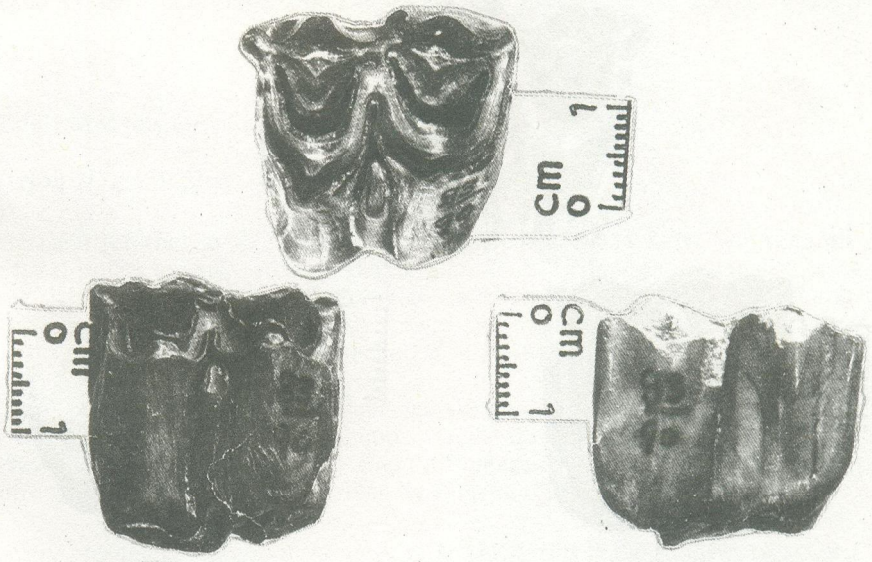

Fig. 2: *Selenoportax lydekkeri*, an isolated left M^1 (P.U.P.C. No. 83/90) from Hasnot, district Jhelum, Punjab, Pakistan. A, Crown view; B, Inner view; C, Outer view.

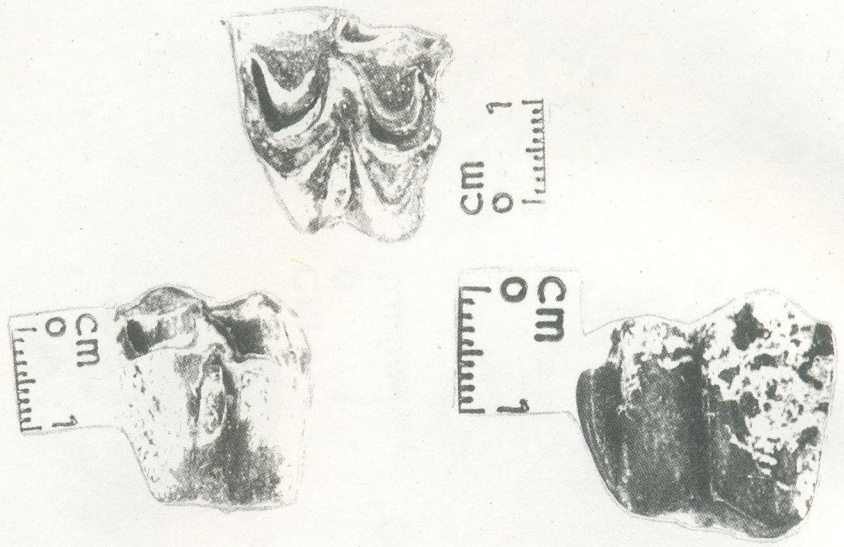

Fig. 3: *Selenoportax vexillarius*, an isolated right M^2 (P.U.P.C. No. 83/93) from Hasnot, district Jhelum, Punjab, Pakistan. A, Crown view; B, Inner view; C, Outer view.

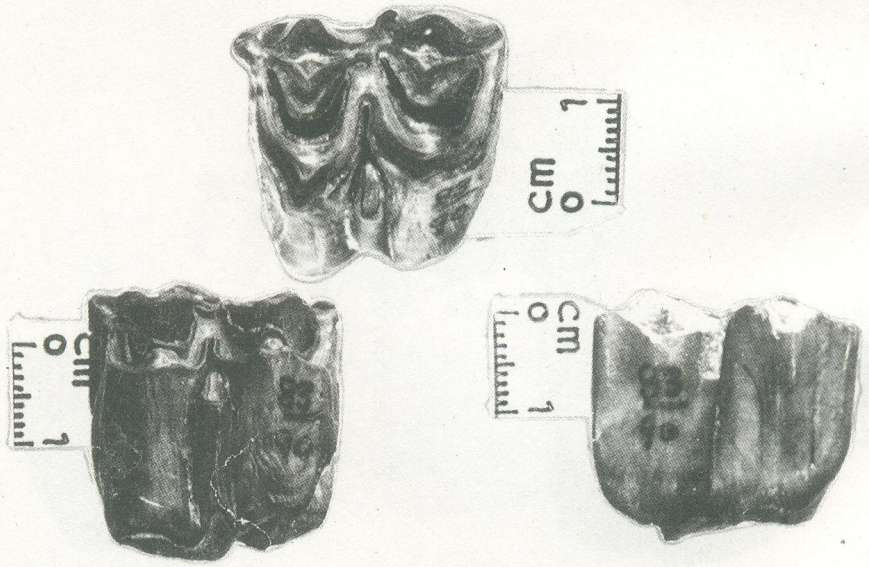

Fig. 4: *Selenoportax lydekkeri*, an isolated right M^2 (P.U.P.C. No. 85/6) from Hasnot, district Jhelum, Punjab, Pakistan. A, Crown view; B, Inner view; C, Outer view.

Material referred to Selenoportax vexillarius

P.U.P.C. (Punjab University [Lahore] Paleontological Collection) No. 83/81 - a damaged left mandible with M_{2-3}.

P.U.P.C. No. 83/82 - a part of right mandible with M_3.

P.U.P.C. Nos. 83/640, 85/8, 85/16, 85/101 - lower molars.

P.U.P.C. Nos. 83/91, 83/93, 83/638, 85/7, 85/9 and 85/12 - isolated upper molars.

Material referred to Selenoportax lydekkeri

P.U.P.C. Nos. 83/90, 85/13 and 85/38 - isolated upper molars.

P.U.P.C. No. 83/84 - a part of left mandible with M_2.

P.U.P.C. No. 83/267 - a left M_3.

Locality

Hasnot, district Jhelum, Punjab, Pakistan for all specimens except P.U.P.C. Nos. 83/640 and 83/638 which come from Dhok Pathan, district Chakwal, Punjab, Pakistan.

Horizon

Middle Siwaliks for all specimens.

Description (Figs. 1-5)

The teeth referred to *S. lydekkeri* are all of left side and fairly worn, in all the median basal pillars are also worn. P.U.P.C. No. 83/13 is damaged internally; it shows an enamel island between the central cavities - a result of advanced wearing. In all the three the antero-external cusp is slightly longer and more flat than the posteroexternal. The width of the teeth diminishes with height. Externally the length increases slightly with height as in Geol. Surv. Ind. molar No. B 206 and American Museum of Natural History (A.M.N.H.) No. 19957 described and sketched by Lydekker (1884) and Pilgrim (1937) respectively. In general, the upper teeth are much larger than those of *S. vexillarius*. The lower teeth show only slight difference of size. This is evident also from the figures drawn and measurements given by Pilgrim (1937).

A very prominent feature of the teeth in *S. vexillarius* is the rugosity of enamel. In some, however, it is less evident but this appears to be due to weathering. The median basal pillar is prominent in all the upper molars. P.U.P.C. No. 83/638 is a right M^2, while the other are very probably M^1. The M^3 (P.U.P.C. No. 83/638) is of an old individual. It compares favourably with A.M.N.H. No. 19844, described by Pilgrim (1937). Its basal pillar is also worn down. The other teeth 83/93 appears to be of a

Fig. 5: Selenoportax vexillarius, a left mandible with M_{2-3} and roots of P_3-M_2 (P.U.P.C. No. 83/81) from Hasnot, district Jhelum, Punjab, Pakistan. A, Crown view; B, Inner view; C, Outer view.

relatively younger individual; it is the right molar. P.U.P.C. No. 83/81 is a left mandible with M_2, M_3 and roots of P_3-M_1. This also shows the same basic features. Like Am. Mus. No. 19514, a median basal pillar is present in M_2 but not in M_3. However, in P.U.P.C. No. 83/82 and 83/640 a median basal pillar is present which suggests that it is a variable feature. The talonid of M_3 is narrow as in A.M.N.H. No. 19514.

Table I: Comparative measurements (mm) of M^1 referred to *Selenoportax vexillarius* and *Selenoportax lydekkeri*.

	S. vexillarius	*S. lydekkeri*	
	83/9*	83/10*	85/38
L	22	27.5	26.6
W	24	28	26
W/L index	109	102	98
H	20	21	27
H/W	83	75	104

Abbreviations used: L, maximum preserved antero-posterior length; W, maximum preserved width of the crown; H, maximum preserved height of the crown.
*The numbers mentioned below the species names are P.U.P.C. Nos.

Table II: Comparative measurements (mm) of M^2 referred to *Selenoportax vexillarius* and *Selenoportax lydekkeri*.

	S. vexillarius								*S. lydekkeri*					
	83/91	83/93	83/116	83/638	85/7	85/12	95/5	96/3	83/90	85/6	85/37	86/201	87/195	95/1
L	25.7	23	23	23	23.5	25	24	25	28.5	29.5	27	28	27.2	28
W	23.8	22.6	20	24	22	26	22	23	28.7	29	27	26.5	27.2	27
W/L index	93	98	87	104	94	104	92	92	101	98	100	95	101	96
H	21	23.4	28	22	22.4	20	27	28	23	28	32	28	25	37
H/W index	88	103	140	92	102	77	123	122	80	96	118	106	91	137

For abbreviations and other details, see Table I.

Table III: **Comparative measurements (mm) of M_1 referred to *Selenoportax vexillarius* and *Selenoportax lydekkeri*.**

	S. vexillarius			*S. lydekkeri*	
	82/98	83/96	96/11	85/26	87/144
L	21	22	20	25.5	27
W/L	15	13	12	12	16
W/L index	71	59	60	47	59
H	16	21.5	19	20	24
H/W index	107	165	158	166	150

For abbreviations and other details, see Table I.

Table IV: **Comparative measurements (mm) of M_2 referred to *Selenoportax vexillarius* and *Selenoportax lydekkeri*.**

	S. vexillarius			*S. lydekkeri*
	83/81	83/84	86/198	85/106
L	27.5	26	29	33.3
W	15	14	13	15
W/L index	54	50	55	45
H	26	26	27	40
H/W index	173	186	208	267

For abbreviations and other details, see Table I.

Table IV: **Comparative measurements (mm) of M_3 referred to *Selenoportax vexillarius* and *Selenoportax lydekkeri*.**

	S. vexillarius			*S. lydekkeri*
	83/81	83/82	86/298	96/8
L	36	36	35	39.5
W	15	14	8	17
W/L index	42	39	23	43
H	30	30	9	27
H/W index	200	214	112	159

For abbreviations and other details, see Table I.

DISCUSSION

Pilgrim (1937 and 1939) based the genus *Selenoportax* upon a collection from the Siwaliks of Pakistan whose type specimen is a skull lacking maxilla and most of basicranium. This is in the collection of the A.M.N.H. No. 19748. Pilgrim (1939) also referred many isolated teeth from Dhokpathan formation, and a left horn-core which was described and figured by Lydekker (1855). Pilgrim (1937) referred all these specimens to the genus *Selenoportax* and added one species in it, *Selenoportax vexillarius*.

Lydekker (1884) described and figured seven different specimens under the *Boselaphus* sp. from "Punjab", no exact locality has been mentioned by him. These specimens were maxilla, mandible and isolated teeth. Pilgrim (1910) established a species *Boselaphus lydekkeri* on this material. However, Pilgrim (1937) transferred all these specimens to the genus *Selenoportax* and species *S. lydekkeri*.

Gentry (1974) proposed that referred teeth of supposed species *Selenoportax lydekkeri* must be regarded as of uncertain identity. Now more material of *S. lydekkeri* has been collected from the Siwaliks by the author. This material comprises, two fragmentary crania, mandibles and many isolated teeth. These specimens are referred to *Selenoportax* in three species, *S. vexillarius, S. lydekkeri* and a third new species *S. dhokpathanensis* (Akhtar, 1992).

In general, the upper teeth of *S. lydekkeri* are much larger than those of *S. vexillarius*. The main difference seen between two species lies in the size of upper teeth, M_3 and mandible. There is no basic difference in the structure of the teeth. The teeth referred to species *S. vexillarius* are comparatively smaller than those of *S. lydekkeri* and are somewhat less hypsodont and have comparatively weaker median basal pillars in the upper mollars.

Similarly, M_3 of the species *S. lydekkeri* is about 40 mm long while those of *S. vexillarius* are 33 or 34 mm long. The other lower teeth show only slightly difference of size (Tables I-V). This is evident also from the figures drawn and measurements given by Pilgrim (1937).

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UPTAKE AND CONCENTRATION OF URANIUM IN ANIMALS AND PLANTS FROM A NATURAL RADIOACTIVE TERRESTRIAL ECOSYSTEM IN PAKISTAN

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Abstract: Radioecological survey was conducted on Miocene-Pliocene rocks of Qabool Khel area, district Bannu. Surface radioactivity ranged from 150 ± 25 to 350 ± 25 counts/sec. Animals and plants collected from the area have been found to contain uranium. Fluorimetric analyses of the grasshoppers of *Acrida* sp., larvae and adults of the butterfly *Anaphaeis aurota*, tenebroid beetles, shells of the mollusc (*Zootecus insularis*), a lizard and a spine of the porcupine *Hystrix indica* showed, 11.57, 2.77, 3.13, 3.03, 0.16, 7.67 and 4.89 ppm of uranium oxide (U_3O_8), respectively. Amongst the plants various species *viz.*, *Calligonum polygonoides*, *Calotropis procera*, *Fagonia cretica*, *Periploca aphylla*, *Peristrophe bicalyculata* and *Rhazya stricta* showed uranium oxide (U_3O_8) contents in the range of 0.03 to 1.25 ppm.

Key words: Radiocontamination, radioactivity, radioecology.

INTRODUCTION

It is well known that animals, plants and microorganisms inhabitants of natural radioactive ecosystems, as well as those exposed to accidental or experimental radioactive environments contain varying degrees of concentrations of radionuclides in their cells/tissues (Garten, 1978; Azam and Prasad, 1989; Juznic and Korun, 1989; Golab *et al.*, 1991; Hyne *et al.*, 1992; Georgescu and Danis, 1994). Radioecological and radiochemical analyses of such biological organisms are of great importance for obtaining information about the environment contamination level, underlying radioactive ore and mineral deposits (Eldridge *et al.*, 1984; Azam and Prasad, 1989; Maksimova, 1996). Besides their role as bioindicators monitoring biological organisms from the contaminated environments for possession of radioactivity is also an important field of study from public health point of view. As, radioactive elements can get entrance to humans by direct exposure to the contaminated environment and/or through a sequence of food chain (Shchalaev *et al.*, 1974; Lungeanu *et al.*, 1993; Chassard-Bouchaud, 1994), depending on amount and exposure time, hazardous effects of radioactive contaminants to human populations include, changes in somatic cells that cause cancer, mutations that affect future generations, effects on embryo and fetus and immediate radiation death (Grace *et al.*, 1980; Hall, 1984; Napolitano *et al.*, 1993).

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There are several natural radioecological areas, harbouring various animal and plant species in Pakistan. There is no information on concentration of radionuclides in these organisms. However, autoradiography of number of animals and plants collected from a radioecological area has indicated the presence of radioactivity (Jafri, 1976). The present study aims at determining the concentration of uranium in various animals and plant species from a natural radioactive terrestrial ecosystem.

MATERIALS AND METHODS

Collection and survey of animals and plants

The survey was conducted on Miocene-Pliocene Siwalik rocks of Qabool Khel area, district Bannu. Surface radioactivity of the study area was measured by a scintillation counter. Small areas of known surface radiation levels were selected for the collection of animals and plants. Animals and plants collected were kept in glass vials and paper files, respectively. No narcotizing agent/preservative was used to kill/preserve the animals. The animals were subjected to death by anaerobic condition in glass vials. The collection was brought to the laboratory as soon as possible.

Fluorimetric determination of uranium

The animal and plant specimens were washed with distilled water to remove clay and sand particles that may contain uranium. They were then dried at 110°C for 24 hours. Each animal or several of one species and different parts of a plant were ground, separately, with the help of pestle and mortar in order to get a fine powder. An amount of 0.2 to 0.3 g of powder was taken in China dishes. The samples were ignited in an electric furnace, at 450°C. The ignited samples were assayed for uranium contents by fluorometric method, as described by Naeem and Abdullah (1984). Briefly described, this method involved leaching of samples in a mixture of HNO_3 and H_2O (1:1) at 250°C for 4 hours. The leached samples were filtered through Whatmann filter paper No.1 and the residue was washed with distilled water. The filtrate was evaporated on hot plate, till it began to dry. Then each sample was dissolved in 50 ml of 0.5N HNO_3. Ten ml of each sample was mixed with 15 ml of saturated Al $(NO_3)_3$ solution and 10 ml of ethyl-acetate. This mixture was poured in separating funnels which were shaken for five minutes in a mechanical shaker. Fifteen minutes were given for clear separation of organic phase (ethyl-acetate) from aqueous phase. One ml of organic phase was taken in clean platinum dishes, which were put under infra-red heaters in a dust free atmosphere in order to dry the organic aliquots. Then with the help of a large size pellitiser, an amount of 3.0 g of mixed carbonate flux (Na_2CO_3, 45.5%; K_2CO_3, 45.5%; NaF, 9.0%) was added in each platinum dish. The material was fused, by keeping the platinum dishes in a fusion assembly for about 15 minutes until a bubble free clear solution was obtained. After 5 minutes of gradual cooling the platinum dishes were removed from the fusion assembly and were placed in a desiccator for 30 minutes. The fluorescence of each pellet was then measured by fluorimeter. Standard of known concentration of uranium oxide (U_3O_8) was also processed, similarly, along with each analysis. The concentration of U_3O_8 in each sample, was measured by the following formula:

U_3O_8 (ppm) = X/S x A/B x C/D x Z/W

where, X = fluorimeter reading of sample; S = fluorimeter reading of standard; A = volume of extractant in ml; B = volume of aliquot of extractant in ml taken in platinum dishes; C = total volume of sample solution; D = volume of aqueous aliquot taken for extraction; Z = weight of U_3O_8 in standard pellet in μg; and W = weight of sample in grams.

RESULTS

Topography of the area

The area is located along eastern fringes of large stream basins known as Bannu basins. The study area mostly consists of low altitude, north-south trending and westerly dipping hills. Surface radioactivity of a small select area ranged from 150±25 to 350±25 counts/second (cps). Arid climate and lack of nutrient rich soil, indicated by rocky and sandy nature, are the major limiting factors of the area. Only two plant species *i.e.*, *Fagonia cretica* and *Rhazya stricta* were abundant. The other species were represented by a few scattered plants. Amongst animals besides the butterflies (*Anaphaeis aurota*) which were quite conspicuous in the area, it was difficult to trace the other animals. Some villages are situated near to the study area and cattles were observed grazing in the area and drinking underground mine water.

Concentration of Uranium in Plants

As the plants; *Fragonia cretica* and *Rhazya stricta* were abundant in the study area, they were uprooted from low (150±25 cps) and high (350±25 cps) radiation areas. The fluorimetric analyses of different parts of these plants revealed that they contained uranium. In general, parts of plants uprooted from the high radiation area contained higher concentration of uranium than the comparable parts of the same species collected from low radiation zone (Fig.1a,b). Concentrations of uranium in other plant species are shown in Fig.1c.

Concentration of uranium in animals

Concentrations of uranium in different parts or whole animal(s) are shown in Fig.1d. Highest concentration of the radionuclide was found in the grasshoppers, *Acrida* sp. Second and third in this order were found, a lizard and a spine of the porcupine, *Hystrix indica*, while very low amount of uranium was detectable in shells of mollusc, *Zootecus insularis*.

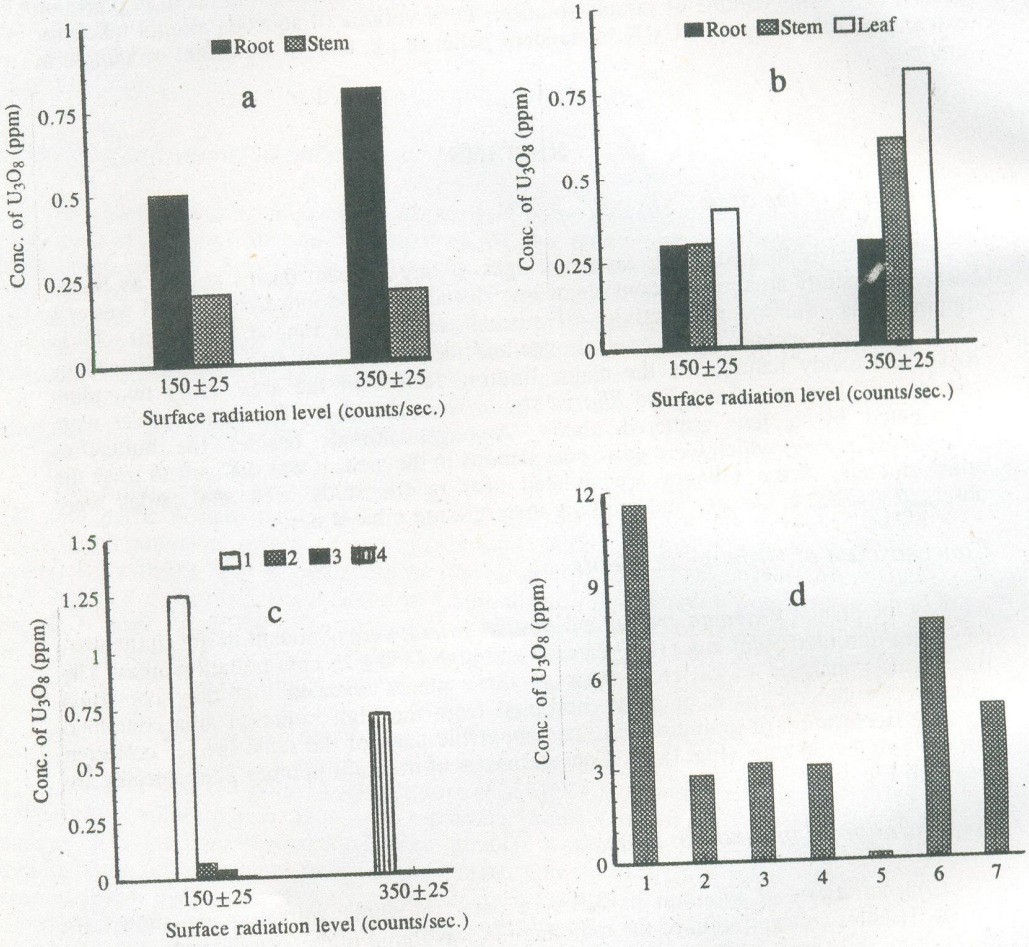

Fig. 1: Concentration of U_3O_8 in; a, different parts of *Fagonian cretica* collected from low and high radiation areas; b, in different parts of *Rhazya stricta* collected from low and high radiation areas; c, in various plants, *Calotropis procera* (1), *Peristrophe bicalyculata* (2), *Periploca aphylla* (3), and *Calligonum polygonoides* (4); and d, in grasshopper of *Acrida* sp. (1), the butterfly's, (*Anaphaeis aurota*) catterpillar (2) and adult (3), tenebroid beetle (4), shells of the mollusc, *Zootecus insularis* (5), a lizard (6) and a spine of the porcupine, *Hystrix indica* (7).

DISCUSSION

Results of this study indicate bioaccumulation and biogenic migration of uranium in animals and plants inhabitants of the area. Concentration of the radionuclide in plants appears to be proportional to the radiation level of soil. This notion has long been established that concentration of an element in a plant is in direct proportion to the concentration of that element in the substrate (Brooks, 1972). Different plant species, uprooted from same radiation area, were found to contain different concentrations of uranium. Similarly, varying levels of radionuclide were detected in different animals, even at the same trophic level. These findings support earlier studies indicating species' differences in bioconcentration of radionuclide in organisms exposed to same radioactive environment. For example, Ahsanullah and Williams (1989) exposed crab, *Pachygrapus laevimanus* and zebra winkle, *Austrocochlea constricta* to uranium (1.5-10 mg/l) in continuous-flow sea water for 40 days and have found that the crabs took up more uranium than winkles; the concentration factors were 7 to 18 and 4, respectively.

Animals (primary and secondary consumers), in general, were found to contain higher concentration of uranium than the concentration at first trophic level. Food chain transport and bioconcentration of various radioactive contaminants including uranium is well documented in literature (Jafri, 1976; Nichols and Scholz, 1989; Driver, 1994).

Further studies are needed to compare differences in genetic make up and other characteristics of such organisms with those inhabiting uncontaminated environments. Concerning potential hazardous impacts of the contamination to local human population, no risk assessment has been evaluated, at least to the knowledge of authors. The cattle observed grazing in the radiocontaminated area may easily be speculated to contain uranium. Future studies should also be extended to this direction.

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CELLULAR COMPONENTS OF THE BRAIN OF *MUSCA DOMESTICA* L. (MUSCIDAE : DIPTERA)

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Abstract: The brain of *Musca domestica* has nervous and non-nervous supporting and nutritive glial tissue. Neurons are for the most part association neurons forming optic, globuli and general type of association or interneurons. Motor neurons are confined to dentocerebrum only and are few. Specialized neurons performing secretory function are abundant in the pars intercenebralis region of the brain. Four types of glial cells are found. The characters and the dimensions vary according to the type.

Key words: Neurons, neurosecretory cells, glial cells, housefly.

INTRODUCTION

Among other things, the survival of every aminal depends on receiving and responding to information about their environment. In addition to this, the monitoring and coordination of internal events is also essential, as different life processes are governed by them. The nervous system performs these integrative functions. It possesses electrical and chemical mechanisum for information reception, transmission, and processing which results in the appropriate behaviour.

In insects the nervous system consists of a chain of central ganglia connected with sensory and effector organs via afferent and efferent nerves. Each ganglion is composed essentially of ramifying neuroglial cells having supporting and nutritive functions and the more distinctive numerous neurous which are specialized for nervous functions and have prologations and branches. Neurons in central nervous systems of insects are characteristically monopolar. Their prologations and branches are processes that are especially adapted for neural transmission and can relatively rapidly generate and conduct electrochemical nervous impulses. (Huber, 1974; Richards and Davies, 1977; Shankland and Frazier, 1985).

Musca domestica, the common housefly was chosen for the present study as it is a mechanical carrier of over hundred species of pathogens causing poliomyelitis, trachoma, infectious hepatitus, Q fever among many other diseases.

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MATERIALS AND METHODS

Collection and maintenance of insects

Musca domestica, used for the present research work were collected from the meat shops near Quaid-e-Azam Campus, Punjab University, Lahore. They were brought to the laboratory where they were sorted out and put in clean 5"x5"x10" plastic jars.

Cotton wool soaked in milk-sugar solution was given as food in small glass petridishes. The flies so maintained were used as stock for rearing purposes. In order to get new generations of files, about 15 mixed adult males and females were taken out and kept in sterilized 3"x6" glass jars. Some rearing was also done in plastic bowls of 8" diameter and 4" depth, containing specially prepared rearing medium which consisted of maize meal, 125 g; molasses, 125 ml; bakers yeast, 18 g; powdered agar, 18 g; propionic acid, 02 ml and water 1075 ml.

The flies usually laid eggs after 24 hours. The eggs hatched after 6-12 hours of deposition. Larval life lasted for about 10 days. Puparial stage lasted for 4-6 days. When the adults emerged, they were kpt in separate containers and fed on a diet of milk-sugar solution.

Histological studies

The head and anterior part of the thorax was removed with the help of a sharp scalpel, fixed for 24 hours in Bouins fixative. Dehydration was done with ascending grades of alcohol, cleared in cedar wood oil and embedded in paraffin wax. Serial 8-10 μm thick sections were cut with a rotary microtome. Sections were stained in hematoxyline, counter stained with eosin, and mounted in canada balsam.

RESULTS AND DISCUSSION

The brain lies just above the oesophagous between the apodemes of the tentorium. It is the dorsal ganglionic centre of the head and is, as usual, made up by the fusion of three embryonic ganglia *i.e.*, Protocerebrum, Deutocerebrum and Tritocerebrum.

Histologically, the two main divisions of the brain are, cortex and medulla or neuropile.

Cortex is the cellular peripheral part possessing three main types of cells *i.e.*, Neurons, Glial or supporting cells and Neurosecretory cells.

Neurons (Figs. 1-7; Table 1)

These cells are the major constituents of the cortex. They lie between the perineurium and neuropile (Richard and Davies, 1977; Ali, 1973, 1993). These are generally categorized as: the motor, sensory and association or internuncial neurons.

This division is based on their morphological and functional characteristics: *e.g.*, size, distribution of chromatin material, presence or absence of nucleoli and whether they perform motor, sensory or association functions constituting transmission channels for the sensory inputs that arrive.

Motor neurons

The only motor neurons found in the brain of the housefly, as in other insects, are present in the region of the deutocerebrum where presumably their axons innervate the mixed antennal nerves. They possess large nuclei with chromatin material concentrated at the centre. Nucleolus is usually single and quite distinct. Considerable cytoplasm is present around the nucleus. Their length ranges from 6-12μm and the width from 6-10μm.

Sensory neurons

These are the commonset type found in the brain and are distinctly recognizable from other types of neurons. They are smaller in size as compared to the other types and form the major bulk of the cortex. They can be divided into three categories according to their location, *i.e.*, optic ganglion neurons; globuli cells of corpora pedunculata; general type composing rest of the sensory cell mass.

a) Optic ganglion neurons

These are small and very tightly packed, so that their cell boundaries are difficult to discern. They have scanty cytoplasm but their nuclei have a rich supply of scattered chromatin granules. Amount of the chromatin material is variable in different cells. Some are more profusely supplied as compared to the others, but in all the cases chromatin rich nuclei almost fill the whole of the cell bodies. The length of their nuclei varies from 2 - 3.5μm, while the breadth ranges from 2 - 2.5μm. Their axons form the different chiasma and axonal tracts of the optic ganglion.

b) Globuli cells of corpora pedunculata

There cells are present on the dorsal side of the corpora pedunculata, filling their cups and calyces and sending their axonal tracts in the neuropile thus forming the stems and roots of the corpora pedunculata. These are also small and very tightly packed like the optic ganglion neurons. They have been called beaker cells by Bretschneider. (1913, 1921 and 1924), globuli cells by Johansson (1957); Ali (1973) and many other later workers. Their nuclei are large and packed with chromatin material. Their cell boundaries could not be clearly distinguished like those of the optic ganglion cells. These cells vary from 3-4μm in length and from 2.5-4μm in width.

c) General sensory cells.

All the neuronal cells of the brain except the above mentioned and association neurons are the 'general sensory cells'. These cells are of variable size. They possess

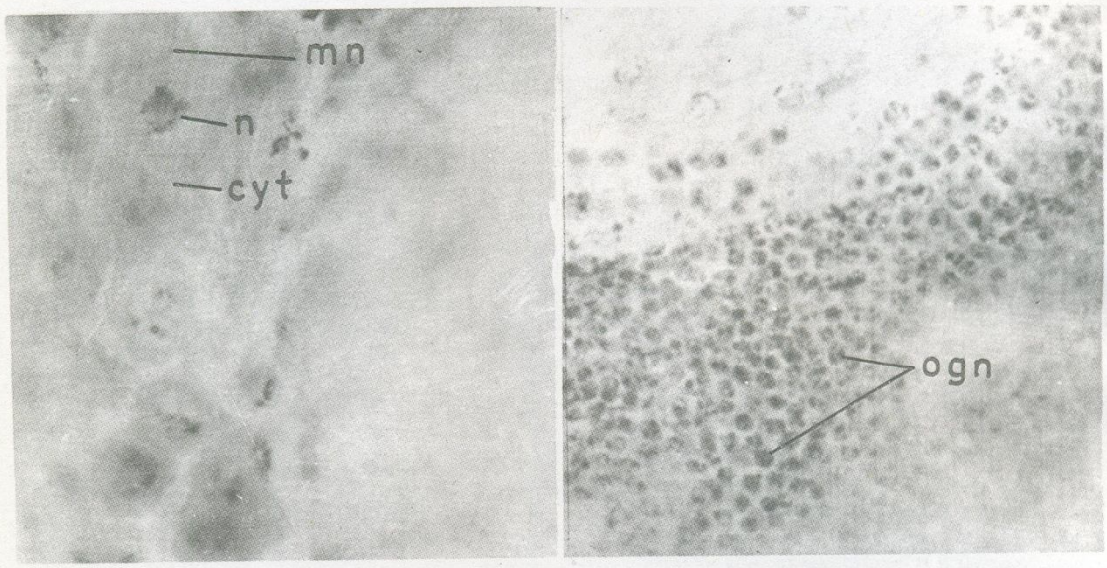

Fig. 1: T.S. of the brain of *Musca domestica*, showing motor neurons (mn), optic ganglion neuron (ogn), cytoplasm (cys) and nucleus (n). Magnification, 100X; Stain, haematoxylin and eosin.

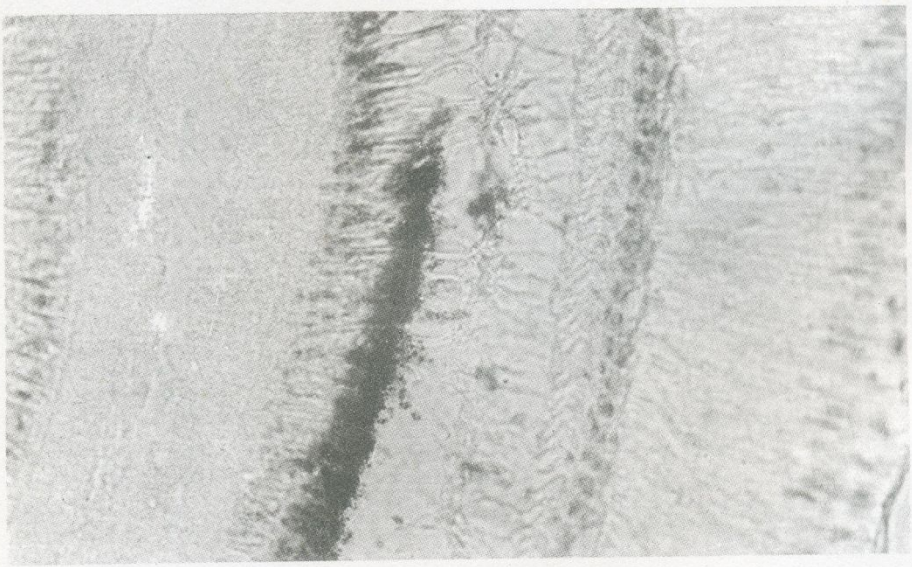

Fig. 2: T.S. of the brain of *Musca domestica*, showing optic lobe. Magnification, 100X; Stain, haematoxylin and eosin.

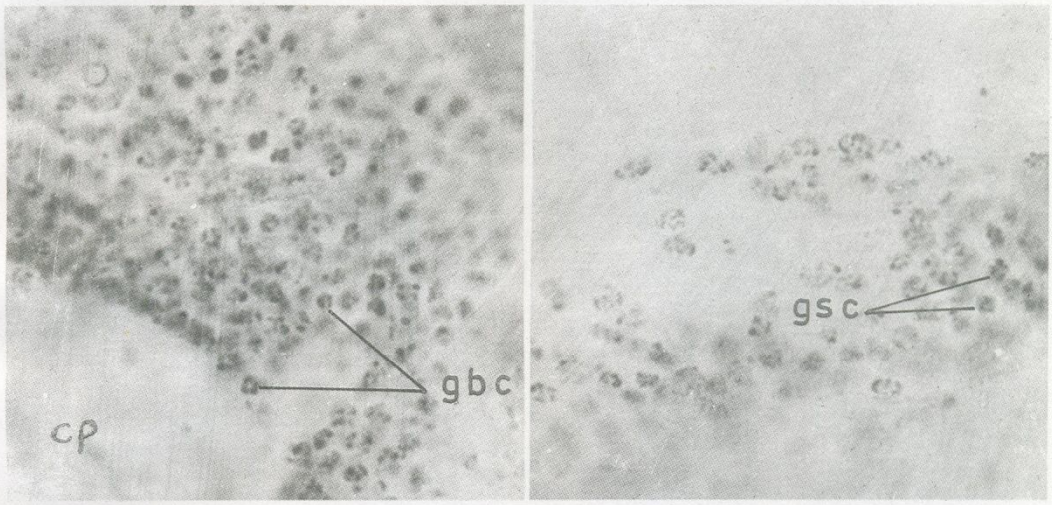

Fig. 3: T.S. of the brain of *Musca domestica*, showing globuli cells (gbc) of corpora pedunculata (cp) and general sensory cells (gsc). Magnification, 100X; Stain, haematoxylin and eosin.

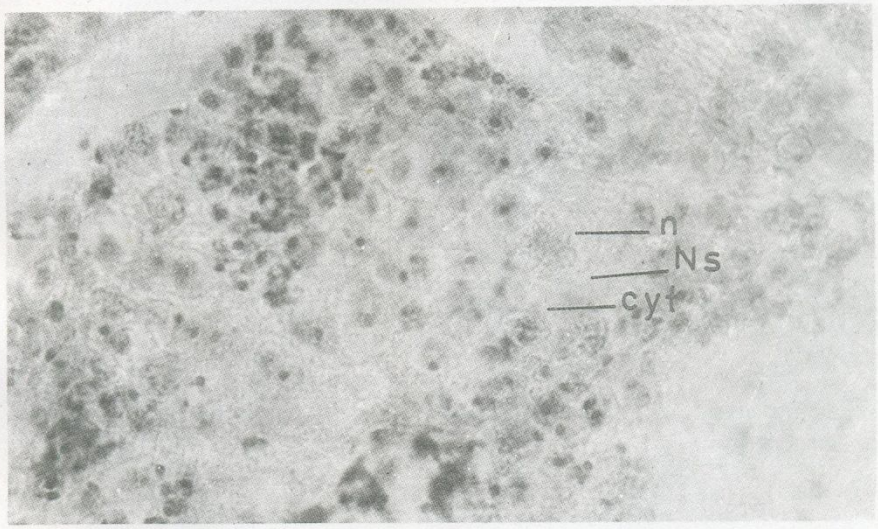

Fig. 4: T.S. of brain of *Musca domestica*, showing neurosecretory cells (ns), cytoplasm (cyt) and nucleus (n). Magnification, 100X; Stain, haematoxylin and eosin.

one or two nucleoli with scattered chromatin material, and have scanty cytoplasm. Their nuclei range from 3-4μm in length and 2.5-3μm in width.

Neurosecretory cells

These are predominantly present in the parts Intercerebralis region of the protocerebrum where they are present in groups. They are specialized neurons which apart from possessing endocrine function also show electrical sings of nervous activity (Cook and Milligan, 1972; Richards and Davies, 1977). All these cells have rounded or elliptical nuclei with a conspicuous nucleolus and centrally concentrated chromatin material. They have abundant cytoplams with distinct cell boundaries. Their nuclei range from 7.5-15μm in length and 5-7.5μm in width. The cell size ranges from 12-20μm in length and 9-20μm in width.

Glial cells.

These form the non-nervous componant of the brain. They have a supporting and nutritional role. Four types of glial cells are present in the cortex and can be recognized as glial type I, type II, type III and type IV. This division was based after the work of Wigglesworth (1956) and Ali (1973).

The glial type I cells

They form the cellular constituents of the perilemma. Their nuclei are small rounded or elliptical. On the dorsal aspect of the brain the perineurium is 1-2 cells thick but on the ventral side and around olfactory lobes it is upto 4 cells thick. The cells are vacuolated with scanty cytoplasm. The thickness of neural lamella, (the outer non-cellular part of the cortex) also varies from 1μm to 5μm. It is thickest at mid-dorsal region and on the ventrolateral aspect of the brain. The length range of nuclei is 5-7.5μm and width range is 2-4μm.

The glial type II cells

These cells are dispersed among the neurons between the perineurium and neuropile. Their nuclei are spherical or elliptical with scattered chromatin material, with a length range of 2.5-5μm and width range of 2.5-5μm. They have distinct cell boundaries but scanty cytoplasm. They are sometimes difficult to distinguish from the neurons.

The glial type III cells

These were easily recognized because of their gaint nuclei. These are elongate with somewhat irregular boundaries. However, their cytoplasm was not discernable. The length of the nuclei ranged from 12.5-17.5μm and width from 9-11μm.

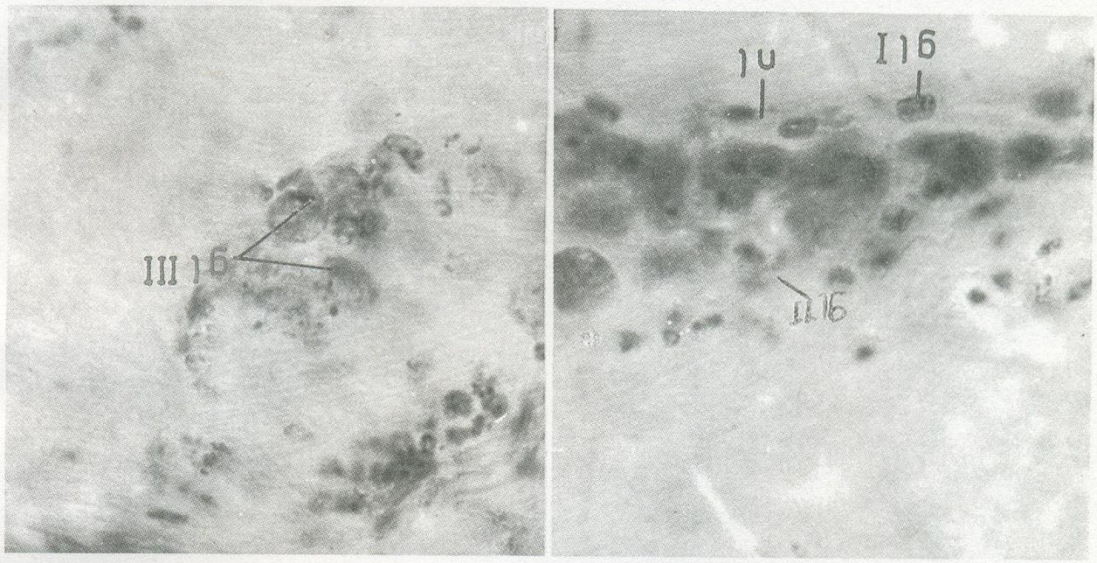

Fig. 5: T.S. of brain of *Musca domestica*, showing neural lamella (nl), glial type I (gl-I), glial II (gl-II) and glial III (gl-III) cells. Magnification, 100X; Stain, haematoxylin and eosin.

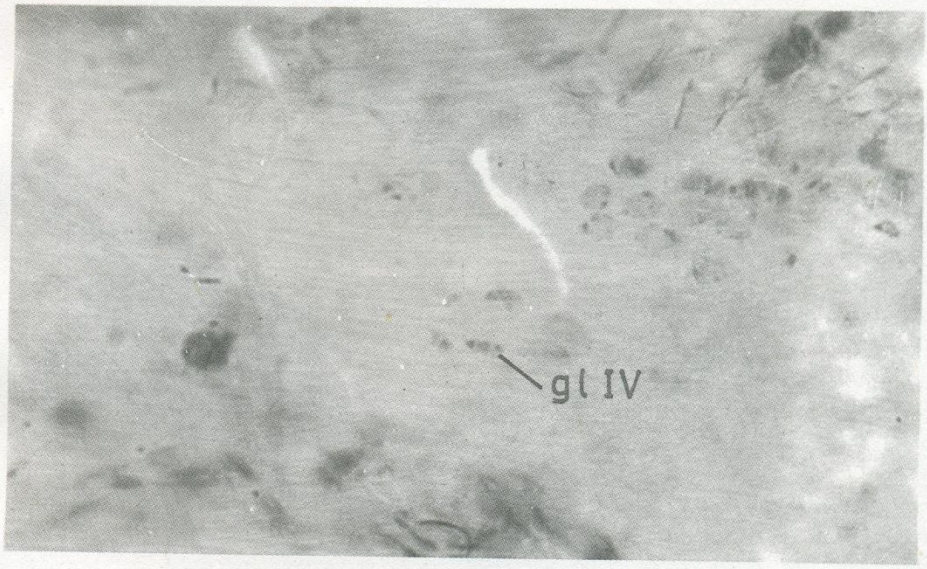

Fig. 6: T.S. of the brain of *Musca domestica*, showing glial IV cells in the neuropile of the protocerebrum. Magnification, 100X; Stain, haematoxylin and eosin.

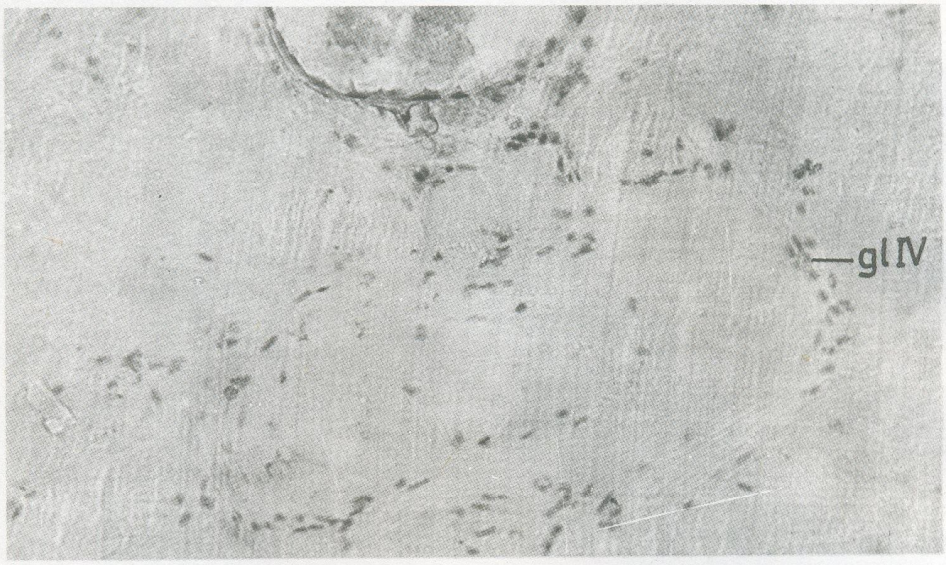

Fig. 7: T.S. of neuropile to the ventral side of protocerebrum showing glial type IV cells and different glomerular bodies. Magnification, 40X; Stain, haematoxylin and eosin.

Table I: Dimensions of nucleic of different cells of brain in control insects of *M. domestica*.

Cell types	Length (μm)			Width (μm)		
	Range	Mean	S.D.	Range	Mean	S.D.
Glial type I	5-7.5	5.5	0.68	2-4	3	0.71
Glial type II	2.5-5	3	0.88	2.5-5	3	0.68
Glial type III	12.5-17.5	15.5	2.73	9-11	10	0.88
Glial type IV	4-5	5	0.55	2-2.5	2	0.27
Motor neurons	6-12	10.5	3.6	6-10	8	1.6
Optic ganglion neurone	2-3.5	2.5	0.55	2-2.5	2	0.31
Globuli cells	3-4	3	0.36	2.5-4	3	0.55
General sensory cells	3-4	3	0.95	2.5-3.5	3	0.32
Neurosecretory cells	7.5-15	10	3.11	5-7.5	6	1.25

The glial type IV cells

These are similar in structure to the glial type I cells. They form a sheath around the neuropile, separating it from the rest of the cortical cells. They also surround all the important glomerular bodies of the brain and are also seen scattered randomly in the neuropile itself. Their cytoplasm is scanty and cell boundaries are indistinct. The nucleus possess scattered chromatin material. The length of the nuclei is 4-5μm and width about 2-2.5μm.

The neuropile

The neuropile is the central part of brain and is traversed by axons of different diameter, some of which are arranged in definite bundles and form distinct fibre tracts. Neuropile also possess some important glomerular bodies for example optic ganglia, cerpora pedunculata and central body. Some of them have been studied in detail (Bullock and Honidge, 1965; Muncini and Frontali, 1967; Frontali and Mancini, 1970 and Weiss, 1974). The neuropile of the olfactory lobe has several clearly distinct glomerular bodies lying in it (Fig.7).

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HISTOPATHOLOGICAL CHANGES INDUCED BY AN ORGANOCHLORINE INSECTICIDE, DDT ON THE LIVER OF ALBINO RAT*

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Abstract: DDT was administered to albino rats as three doses (100 mg, 20 mg and 10 mg/kg B.Wt./day) for 48 hours, 15 days and 18 months durations, respectively. Animals were dissected after the stipulated periods, liver samples were removed and processed for histological analyses. Hepatic cell hypertrophy was the most common structural change induced by all doses of DDT. During 48 hour treatment, nuclear condensation, hyperchromatic and irregular shaped nuclei were observed. Clear zones around the nuclei and disorganized hepatic cord structure were also prominent changes. In 15 day DDT feeding dilation of sinusoidal spaces, enlarged blood vessels and bile canaliculi hyperchromatic nuclei, with increase in kupffer cells were noticed. Alteration in hepatic cords were also prominent in 6, 12 and 15 day treatments. During 18 month DDT feeding study somewhat degenerated (necrotic) zones alongwith irregular shaped hyperchromatic nuclei were observed. Highly hypertrophied kupffer cells were also present. Fatty degeneration and increased thickness of the cell membranes were also noticed in 12 and 18 months DDT treatments. Among the morphometric studies the increase in hepatic cell size, with decrease in number of cells/microscopic field with occasional rise in nuclear and nucleolarize were important findings in all three DDT feeding studies.

Key words: Hepatic morphology, morphometric changes, liver histology.

INTRODUCTION

Insecticides, heavy metals and chemical effluents from the industries are significant sources of pollution in the present world (Radulescu *et al.*, 1990; Shahida and Solangi, 1990; Jabbar *et al.*, 1991; Winter and Street, 1992; Rani *et al.*, 1993; Sukhoparova *et al.*, 1994; Vuorinen *et al.*, 1994; Urdaneta *et al.*, 1995). Among these pollutants, organochlorine group of insecticides is more dangerous as far as the environmental damage and degradation is concerned. DDT is one of these important organochlorine compound, which has been extensively used for controlling the pests of agriculture and public health importance (Metcalf, 1973; Floodstrom *et al.*, 1990; Gecheva, 1991; Bhatnagar *et al.*, 1992; Douthwaite, 1992; Aboul Ela *et al.*, 1993; Mourya *et al.*, 1993; Galassi *et al.*, 1994; Dirksen *et al.*, 1995).

Large amount of residues of DDT have constantly been detected and reported from

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all facets of life after its use for few years. The major cause of development of these residues was the persistant and highly stable nature of organochlorine compounds including DDT (Mohammad *et al.*, 1990; Calero *et al.*, 1992; Rao, 1992; Singh *et al.*, 1992; Mason and Madsen, 1993; Thao *et al.*, 1993).

Number of xenobiotic compounds have the ability to induce various type of pathologies in animal tissues. Organochlorine compounds are of great significance among those (Roberts and Robert, 1982; Kandalaft *et al.*, 1991; Jonsson *et al.*, 1993; Keith *et al.*, 1993; Katayama, 1993; Teshke *et al.*, 1993; Srivastava and Srivastava, 1994). These compounds also produced hepatotoxic effect, which has also been reported from this and other laboratories (Shakoori *et al.*, 1982, 1984; Ali *et al.*, 1988; Gupta *et al.*, 1989; Ali and Shakoori, 1990, 1993; Bagchi *et al.*, 1993; Misutani *et al.*, 1994; Begum and Vijayara-ghavan, 1995).

Conflicting reports about the carcinogenic potential of DDT are also found in literature (Turusov *et al.*, 1973; Kashyap *et al.*, 1977; Rossi *et al.*, 1977; Flodstrom *et al.*, 1990; Yusof and Edwards, 1990; Adenuga *et al.*, 1992).

The objective of present report was to study the histopathological and morphometric effects of various sublethal doses of DDT on liver, administered for short and long term durations.

MATERIALS AND METHODS

Sprague Dawley rats reared in the Animal House of Department of Zoology were used for the experiment. About 5 month old animals were used for short term experiments, while younger rats about 3 months old were used for long term experiment.

Five to six animals were caged in 2.5 cubic feet iron cages and provided with food and water *ad libitum*. Laboratory prepared rat feed was used for the study, the composition of which has already been mentioned (see, Ali and Shakoori, 1990).

Administration of insecticide

An insecticide belonging to chlorinated hydrocarbon group, DDT (1,1,1-trichloro-2,2-bis (4-chlorophenyl ethane), collected as 75% powder from Agriculture Department, Government of the Punjab, was used for this study. The toxicant was administered to animals alongwith the feed as three different doses for variable durations *i.e.*, 100, 20 and 10 mg/kg body weight/day for 48 hour, 15 day and 18 month durations, respectively. In first short term (ST-I) experiment, 800 mg DDT (75%) was added per kg of dry feed. In second case (ST-II experiment) 525 mg of DDT was mixed with 3 kg of feed while in long term (LT) case, 87.5 mg of DDT was thoroughly mixed per one kg of dry rat feed, with small amount of water to prepare thick cakes of feed.

Procedure adopted

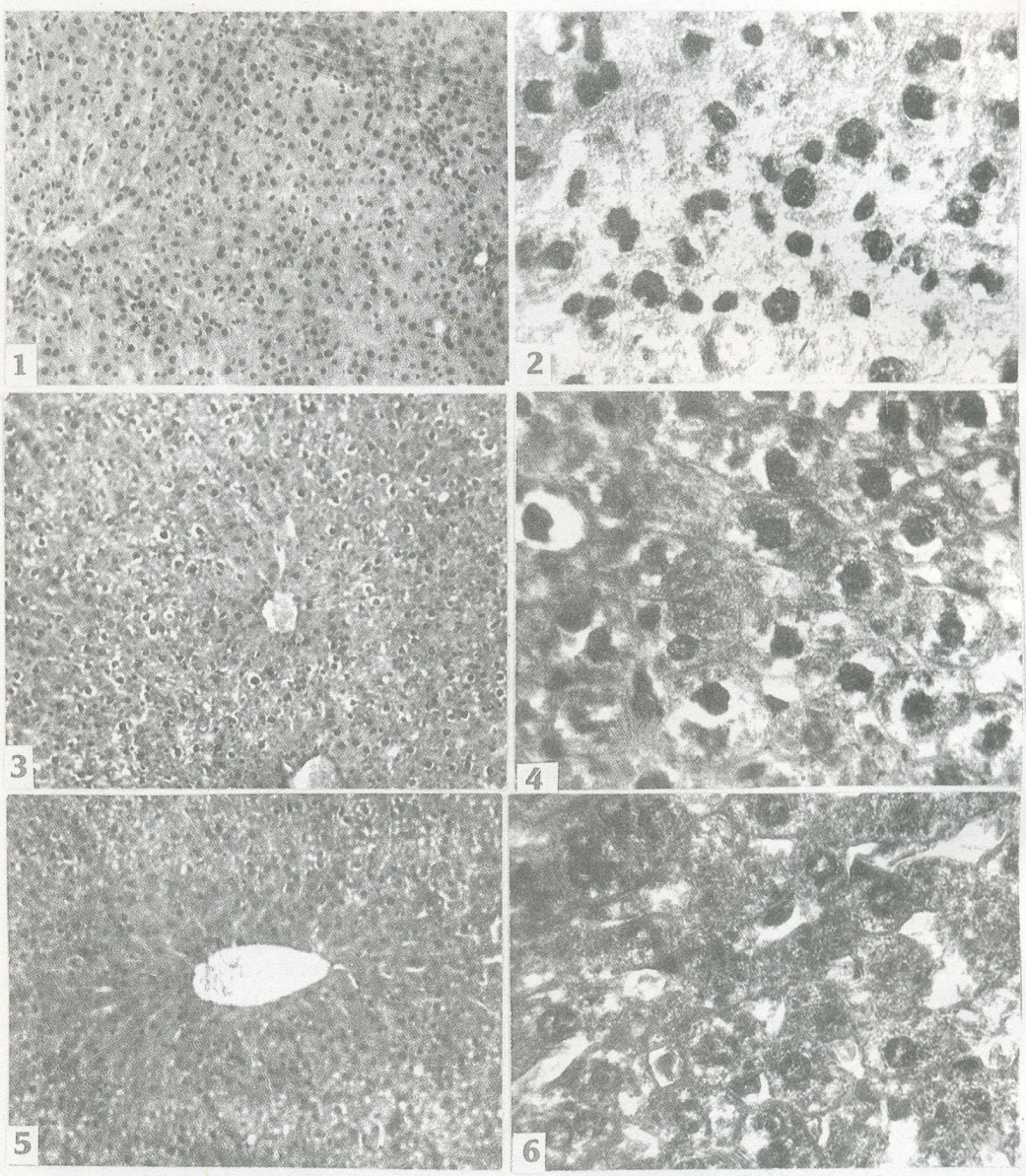
A strong dose of DDT (100 mg/kg B.Wt./day) was fed to a group of 4 rats for short term (48 hours) duration. Two weak doses (20 mg and 10 mg/kg/day) were administered to two other groups of rats for 15 days and 18 months, respectively. A control group was also treated similarly, except insecticide treatment for each experiment.

After the stipulated periods of 24 and 48 hours in strong dose experiment, after 3, 6, 9, 12 and 15 days in 1st week dose case and after 6, 12 and 18 months duration in 2nd week dose experiment, 3-6 animals were anaesthetized and dissected from both DDT treated and control groups. Their liver samples were quickly removed and fixed in Bouin's fixative.

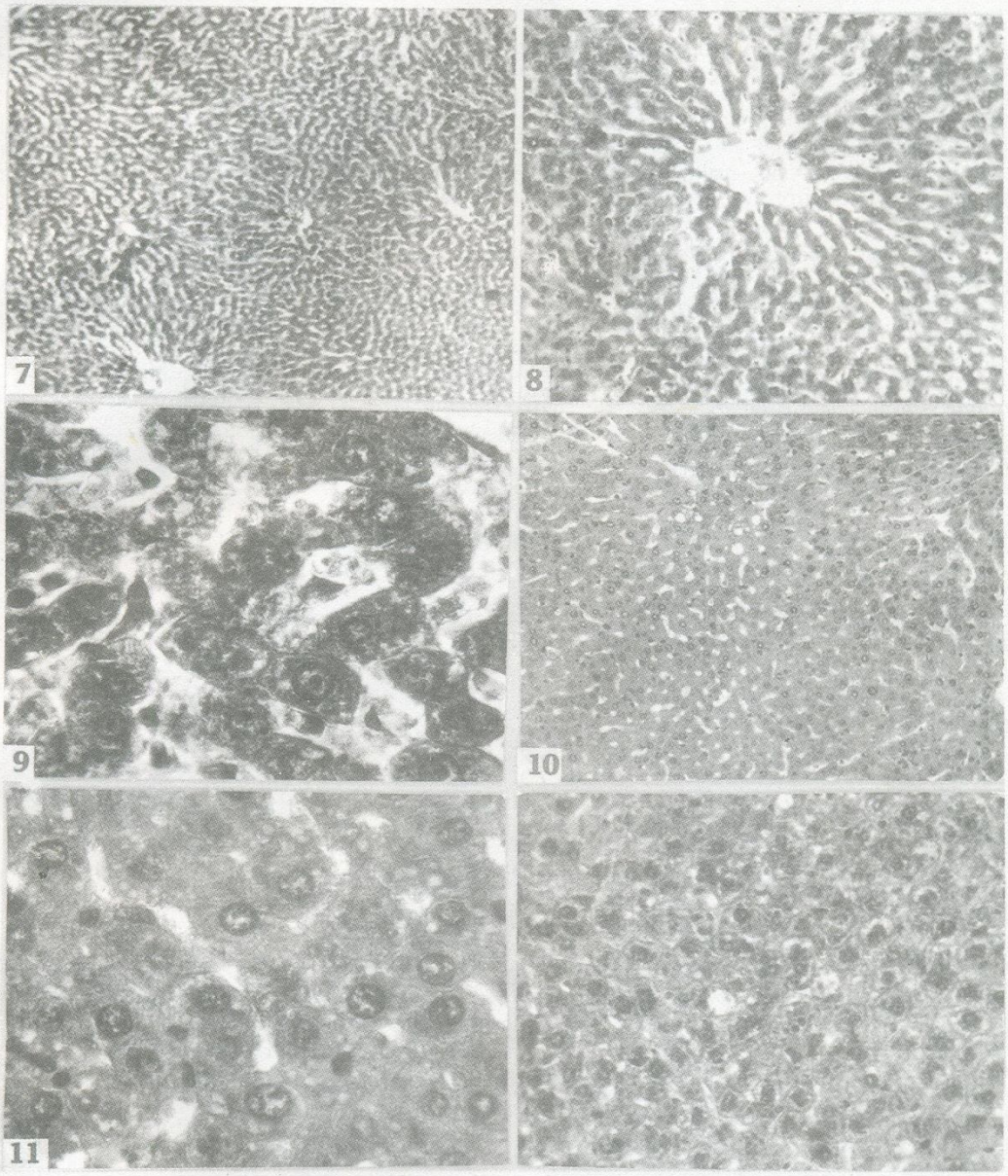
The liver samples were further processed for microtomy using routine histological techniques. About 6-8 μm thick liver sections were prepared and stained with haematoxyline and eosin differential staining technique, which were further studied for various histological changes. The liver sections were also studied for different morphometric parameters, which include the size of hepatic cell, nuclei and nucleoli, number of cells per microscopic field, number of nuclei per cell and number of nucleoli per nucleus. Ocular micrometer was used for measuring the size of cell and its components which was initially calibrated with stage micrometer.

RESULTS

Tables I-III and Figures 1-24 show the effect of various doses of DDT (100 mg, 20 mg and 10 mg/kg/day) on the morphometric and histological structure of liver. The hepatic cells increased in size in both short-term treatments. This increase was 29% in 24 hours feeding group and 23% in 48 hour feeding case. In second ST experiment this increase was 14-26%. In 18 months (long-term) DDT treatment similar change in hepatic cells was also found with 27-45% increase. The nucleolar size in this experiment increased significantly which was 56% in 24 hour and 44% in 48 hour feeding group. The number of nuclei/cell and number of nucleoli/nucleus remained unaffected in all three treatments. The number of cells/field showed significant decrease during both ST experiments. This decrease was 16 and 22% during 48 hours and 17-28% in 15 day treatments. The size of the nucleus remained unchanged in first ST experiment while in second ST experiment when it was increased by 18% and showed 13% rise in 15 day treatment. The nucleolar size did not show any significant change except 29% increase at day 3 in 15 day DDT feeding experiment. Table III shows the effect of long term feeding of DDT on the various histological parameters of rat liver. As is typical of all the chlorinated insecticide toxicity in non-target organisms, the hepatic cells showed hypertrophy. The hepatic cell size increased 33%, 27% and 45% with simultaneous decline in number of cells/microscopic field with 22, 21 and 29% decrease after 6, 12 and 18 months of feeding, respectively. The nuclear size of hepatic cells increased significantly (20%) after 18 months of insecticide feeding. The nucleolar size, on the other hand, remained unaltered.



Figs.1-6. Histological structure of normal rat liver showing hepatic sinusoidal spaces (Fig.1), normal hepatic morphology (Figs.1-2). The hepatic structure of DDT-fed animals for 24 hour (Figs.3-4) and 48 hours (Figs.5-6) are also shown. Note disturbed sinusoidal areas and hepatic cords (Fig.3), clear areas in the cytoplasm around nuclei and hypertrophy of hepatocytes (Figs.3-4), darkly stained oval area around central vein, and fine clear areas in the tissues (Fig.5), hypertrophied cells, nuclei with large clear areas around nucleus and in cytoplasm (Fig.6). Magnification: Figs. 1, 3, 5, 50X; Figs. 2, 4, 6, 250X. Stain: haematoxylin and eosin.



Figs.7-12. Histological structure of rat liver fed on DDT-mixed diet for 3 days (Figs.7-9), 6 days (Figs.10-11) and 9 days (Fig.12). Note altered lobular morphology (Figs. 7-10), increase in number of kupffer cells (Figs. 8-9), enlarged sinusoidal areas (Figs. 8, 9, 11), cellular and nuclear hypertrophy (Figs.9-10) and slight vacuolation (Figs.10-12). Magnification: Fig.7, 25X; Figs. 8, 10, 50X; Fig. 12, 100X; Figs. 9, 11, 250X; Stain: haematoxylin and eosin.

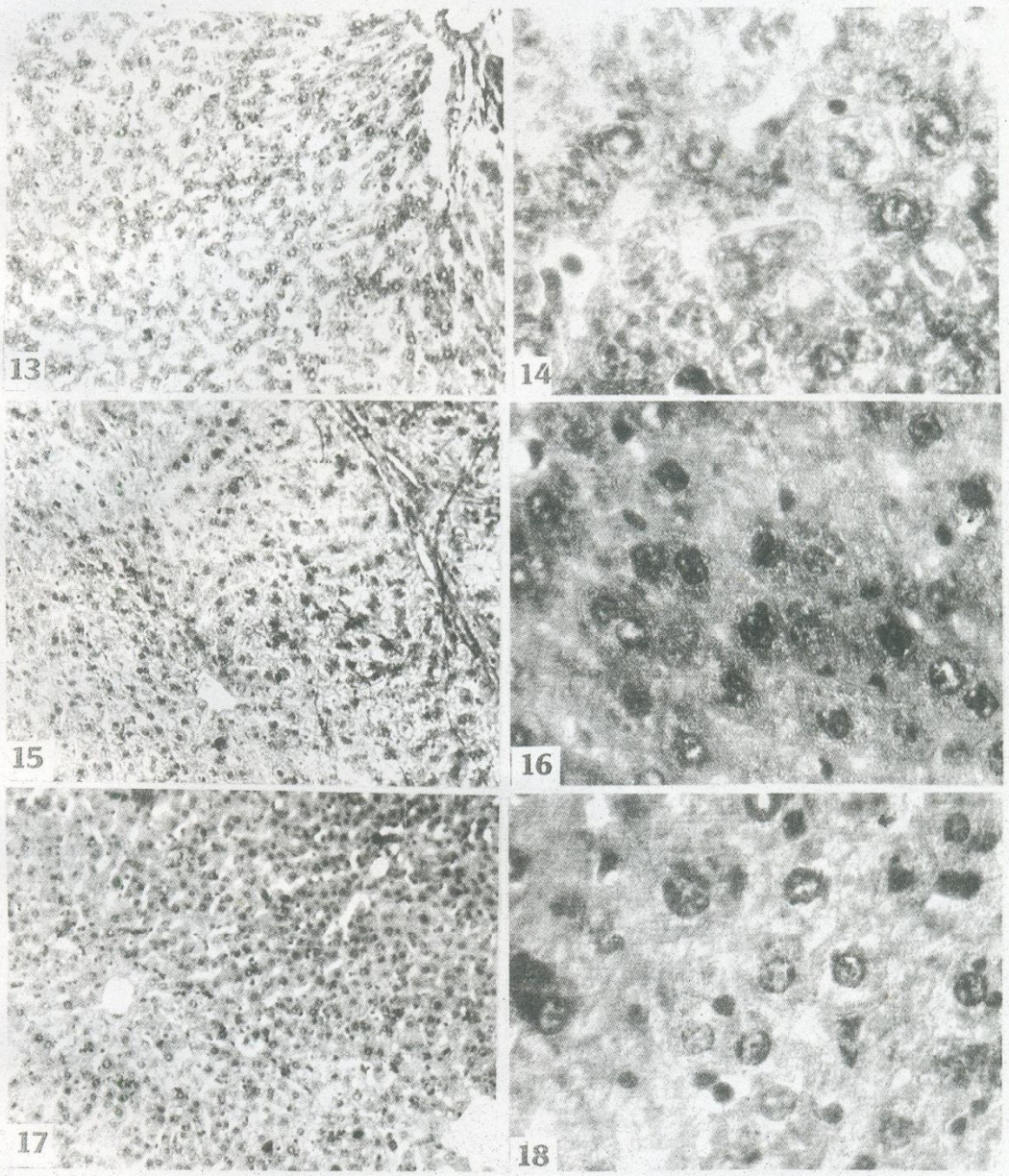
Figures 3 to 5 showed histological structure of liver of rat fed on DDT mixed diet for 24 hours, while Figures 5 and 6 showed histological changes after 48 hours of DDT feeding when compared with control liver (Figs.1-2). The hepatic cells and nucleoli showed hypertrophy. The nuclei in 24 hour group were condensed with a clear space around them. The blood vessels and bile canaliculi were prominently enlarged (compare Figure 3 with Figure 1). In 48 hour treatment the nuclei were distinctly prominent and vesicular with irregular clear zones in the tissue (Fig.6). The general hepatolobular architecture showed abnormalities and necrotic region on the periphery (Figs.3-6), when compared with control liver structure (Figs.1-2).

Prominent changes in hepatic structure of rat were observed following DDT feeding for 3 days (Figs.7-9) 6 days (Figs.10-11), 9 days (Fig.12), 12 days (Figs.13-14) and 15 days (Figs.15-16). The hypertrophied cells, well defined vesicular nuclei enlarged kupffer cells and sinusoidal areas (Figs.9, 11, 14, 16, 18) swollen bile canaliculi and other blood vessels (Figs.7-8, 17) when compared with control (Figs.1-2) were the prominent structural changes in liver with increasing duration of DDT administration. The cellular hypertrophy and formation of vesicular nucleus (Figs.11, 16, 18) became more prominent and disorganized lobular zones with disruption of hepatic cord structure (Figs. 10 and 12-15) and fatty degeneration (Figs.11-13) were further increased with increase in duration of treatment.

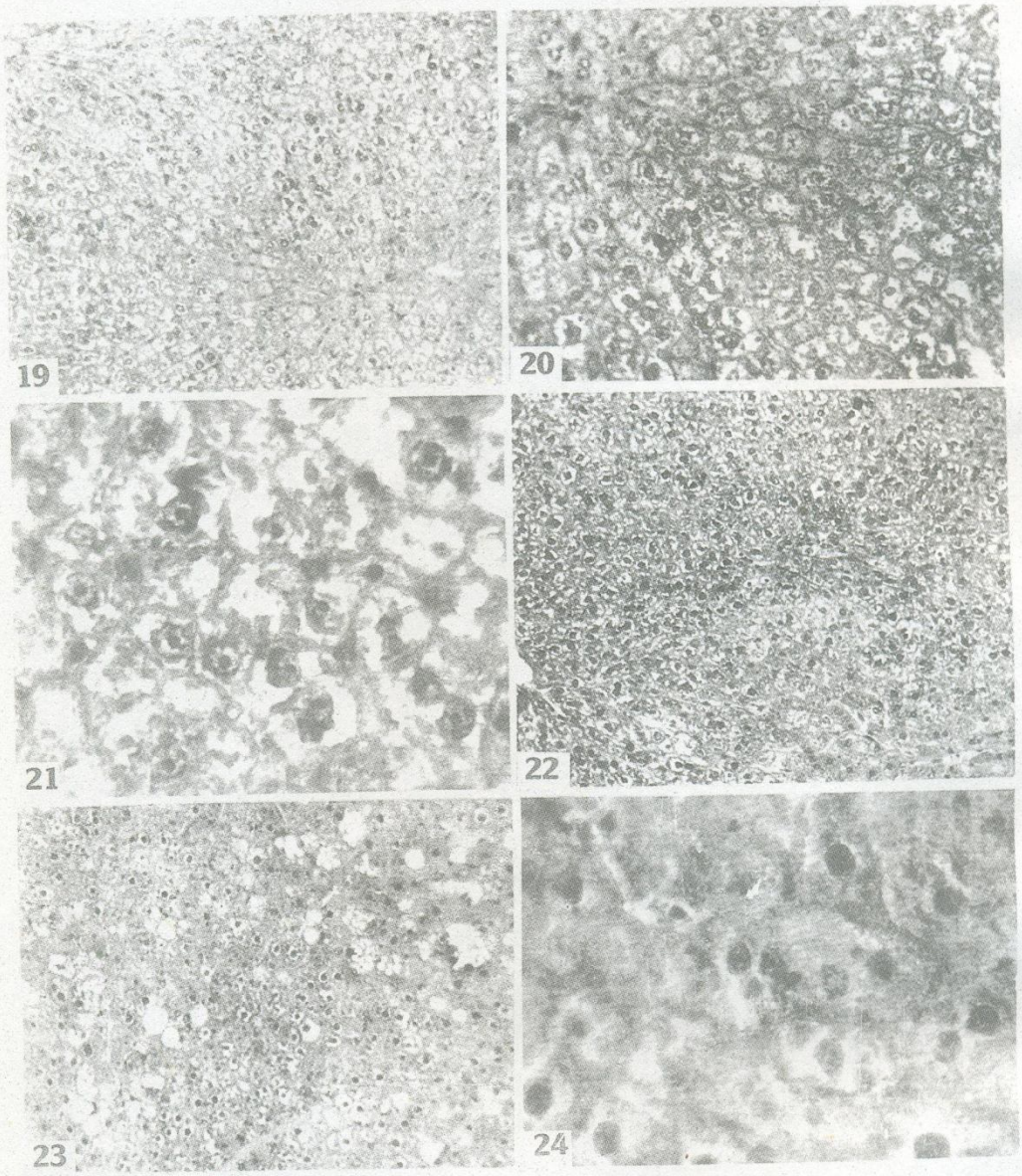
Table I: Effect of feeding DDT-mixed diet (100 mg/kg body weight/day) for 48 hours on various histological parameters of rat liver.

Parameters	Control	DDT-fed	
		24 hours	48 hours
No. of cells/ field (n=9)	291.42[a] ±16.73	244.55* ±9.78	228.49** ±13.52
No. of nuclei/ cell (n=90)	1.10 ±0.08	1.15 ±0.05	1.18 ±0.07
No. of nucleoli/ nucleus (n=90)	1.58 ±0.16	1.78 ±0.25	1.72 ±0.16
Size of cell (μ^2; n=90)	281.77 ±11.46	363.72*** ±9.02	345.57*** ±6.30
Size of nucleus (μ^2; n=90)	39.57 ±2.04	37.54 ±1.18	40.98 ±1.34
Size of nucleolus (μ^2; n=90)	2.52 ±0.34	3.93** ±0.38	3.62* ±0.26

Mean ± SEM. Student's 't' test; *: P<0.05; **: P<0.01; ***: P<0.001.



Figs.13-18. Histological structure of rat liver fed on DDT-mixed diet for 12 days (Figs.13-14), 15 days (Figs.15-16) and 6 months (Figs.17-18). Note highly disorganised cord morphology (Figs. 13, 15, 17) fairly abnormal sinusoidal areas with numerous round and swollen kupffer cells (Figs. 13, 16, 17, 18), hypertrophied (Figs. 14, 18), irregular shaped condensed nuclei (Figs. 15-17) and distorted blood vessels (Figs. 15, 17). Magnifications: Figs. 13, 15, 17, 50X; Figs. 14, 16, 18, 250X; Stain: haematoxylin and eosin.



Figs.19-24. Histological structure of rat liver fed on DDT-mixed diet for 12 months (Figs.19-21) and 18 months (Figs.22-24). Note disturbed lobular and cord morphology distinctly prominent membrane network (Figs. 19-20), cytoplasmic margination (19-23), extensive vacuolation (Fig.23), hypertrophied (Fig.21) and irregular shaped condensed nuclei (Figs. 20, 21, 24). Magnification: Figs. 19, 22, 23, 50X; Fig. 20, 100X and Figs. 21, 24, 250X. Stain: haematoxylin and eosin.

Table II: Effect of feeding DDT mixed diet (20 mg/kg body weight/day) for 15 days on the various histological parameters of albino rat liver.

Parameters	Control	DDT-fed				
		3 days	6 days	9 days	12 days	15 days
No. of cells/ field (n=9)	267.44[a] ±15.13	242.39 ±11.19	221.29* ±9.48	232.61 ±12.77	205.61** ±10.37	193.48*** ±9.87
No. of nuclei/ cell (n=90)	1.12 ±0.04	1.25 ±0.06	1.15 ±0.07	1.12 ±0.04	1.19 ±0.04	1.23 ±0.09
No. of nucleoli/ nucleus (n=90)	1.58 ±0.15	1.60 ±0.14	1.82 ±0.13	1.80 ±0.24	1.70 ±0.11	1.80 ±0.25
Size of cell (μ^2; n=90)	270.63 ±11.21	298.36 ±8.05	308.18** ±7.80	309.12** ±6.25	334.82*** ±8.05	341.05*** ±15.13
Size of nucleus (μ^2; n=90)	43.34 ±1.20	45.40 ±1.75	44.36 ±1.47	46.98 ±1.56	51.09*** ±1.55	48.87** ±1.35
Size of nucleolus (μ^2; n=90)	2.87 ±0.21	3.70* ±0.25	3.35 ±0.23	3.26 ±0.36	3.80 ±0.45	3.09 ±0.60

[a]Mean ± SEM. Student's 't' test; *: P<0.05; **: P<0.01; ***: P<0.001.

On comparison of Figures 17-18 (6 month feeding), 19-21 (12 months feeding) and 22-24 (18 months feeding) with figures 1-2 (control group) several histological changes can be observed. Although the general hepatolobular architecture was maintained the hepatic cells showed hypertrophy (Figs.20, 21, 24) after 12 and 18 months of DDT feeding. The nuclei became well demarkated irregular shaped and condensed (Figs.17, 20 and 22). The plasma membranes of hepatic cells and bile canaliculi also became very prominent (Figs.20-21). Eighteen and twelve months of DDT feeding also resulted in appearance of numerous vacuoles in the hepatic tissue (Figs. 20, 21 and 23) which could be fatty degeneration of hepatic tissue and are indicative of its toxicity. The hepatic nuclei also became very much condensed and were surrounded by a clear zone (Figs. 19-23).

Table III: Effect of feeding DDT mixed diet (10 mg/kg body weight/day) for a total period of 18 months on the various histological parameters of rat liver.

Parameters	6 months		12 months		18 months	
	Control	DDT fed	Control	DDT fed	Control	DDT fed
No. of cells/ field (n=9)	238.37[a] ±11.68	184.78** ±10.49	269.41 ±12.40	213.90** ±14.41	244.64 ±10.71	174.81** ±13.81
No. of nuclei/ cell (n=90)	1.07 ±0.06	1.17 ±0.08	1.11 ±0.04	1.17 ±0.08	1.04 ±0.03	1.12 ±0.04
No. of nucleoli/ nucleus (n=90)	1.52 ±0.16	1.88 ±0.31	1.46 ±0.14	1.63 ±0.21	1.40 ±0.19	1.62 ±0.12
Size of cell (μ^2; n=90)	290.31 ±8.78	387.12*** ±15.76	258.61 ±14.17	328.34*** ±6.84	273.44 ±11.19	395.56*** ±11.37
Size of nucleus (μ^2; n=90)	37.91 ±1.23	40.96 ±1.50	41.72 ±1.81	45.92 ±1.57	43.69 ±1.94	52.26* ±2.88
Size of nucleolus (μ^2; n=90)	2.79 ±0.21	3.29 ±0.22	2.94 ±0.30	3.24 ±0.32	3.11 ±0.24	3.50 ±0.39

[a]Mean ± SEM. Student's 't' test: *: P<0.05; **: P<0.01; ***: P<0.001.

DISCUSSION

The DDT feeding results in hypertrophy of hepatic cell and increase in its nuclear and nucleolar size. In 48 hour feeding experiment the cell size showed about 23% increase, while nucleolus showed 44% increase after 48 hours of DDT feeding. In 15 day feeding experiment the cell size increased upto 26% while nucleus showed 18% increase. In 18 month feeding experiment, this increase was respectively, 45% and 20%. The number of cells per microscopic field decreased by 22%, 28% and 29% at 48 hours, 15 days and 18 months feeding experiment. The number of nuclei and nucleoli remained unaltered.

Besides these changes in size of hepatic cell and its components, typical hepatic damage, as manifested in the form of sinusoidal congestion, cellular vacuolation and foamy appearance, was evident in the present study. Similar type of morphological changes have been reported from other labortories (Datta and Dikshith, 1973). Ramalingam (1985) has found harmful effects of DDT on liver structure which may lead to alterations in tissue metabolism. DDT treatment produced moderate fatty

Table IV: **Effect of feeding DDT mixed diet for three different durations on the various histological parameters of rat liver. The values are shown as percent increase (+) or decrease (-) with reference to their respective controls.**

Parameters	DURATION OF DDT TREATMENT									
	Hours		Days					Months		
	24	48	3	6	9	12	15	6	12	18
Cell size	+29***	+23***	+10	+14**	+14**	+24***	+26***	+33***	+27***	+45***
Nuclear size	-5	+4	+5	+2	+8	+18***	+13**	+8	+10	+20*
Nucleolar size	+56**	+44*	+29*	+17	+14	+32	+8	+18	+10	+13
No. of cells/ field	-16*	-22**	-9	-17*	-13	-23**	-28***	-22**	-21**	-29**
No. of nuclei/ cell	+5	+7	+12	+3	-	+6	+10	+9	+5	+8
No. of nucleoli/ nucleus	+13	+9	+3	+17	+15	+9	+15	+24	+12	+16

* P.0.05; **: P<0.01; ***: P<0.001 (Student's 't' test).

degeneration, ultrastructural changes in the mitochondria, endoplasmic reticulum and lysosome in rat liver with maximum on day 60 (Kaloyanova-Simeonova *et al.*, 1983). It has been concluded in this study that after day 90, the adaptive process restricts the further damage. Kimbrough *et al.* (1971) have shown that 250 ppm DDT dose, given to rats, caused enlargement of the hepatic cells around the central veins and cytoplasm has smooth appearance. The dietary concentration of 500 ppm caused cytoplasmic inclusions in number of hepatic cells and the enlargement of the cells (hypertrophy) had spread to a larger portion of the liver lobules. Margination and moderate number of vacuolated cells were also observed at this dose. These findings can also be correlated with the present studies. Similar changes in hepatic structure following oral administration of other organochlorine insecticides has already been reported from this laboratory (Ali and Shakoori, 1990 1993). DDT also induced changes in other tissues of animals. Baronia and Sahal (1993) reported induction of necrosis in seminiferous tissue hypertrophy in germinal epithelium of sperm cells shrinkage of spermatogonia, spermatocytes and spermatids following DDT feeding (@ 500 mg/kg B.Wt.) for six weeks in albino rats. Although, DDT-induced carcinogenic changes in liver were not reported during this study. There are considerable evidences that DDT proved carcinogenic in different animals (Thorpe and Walker, 1973; Kashyap *et al.*, 1977), while Laws (1971) concluded that DDT has antitumorigenic properties in a controlled experimental tumour system.

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MULTIPLE ANTIBIOTIC RESISTANCE AMONG *SHIGELLA* SPECIES ISOLATED FROM PATIENTS OF SHIGELLOSIS IN PAKISTAN

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Abstract: Twenty-four Shigellae strains were isolated from different clinical sources in Pakistan. These isolates were screened for their resistance to the antibiotics, ampicillin, chloramphenicol, enoxacin, gentamicin, septran, tetracycline, urixin and velosef. Minimum inhibitory concentrations were determined and the isolates showed the highest frequency of resistance against Septran at 50 and 100 μg/ml whereas at 250 and 500 μg/ml, the highest frequency of resistance was against Urixin. The lowest frequency of resistance was against Enoxacin, followed by gentamicin and Chloramphenicol. At 250 μg/ml level, the isolates showed a considerable decrease in the resistance frequency of almost all antibiotics. In the present study, 95.8% of these Shigellae were multi-drug resistant strains. Our results emphasize the urgent need to exercise restraint in the use of oral antibiotics in Shigellosis. The overall incidence of *Shigella* infections can be decreased by improvement in the levels of environmental and personal hygiene.

Key words: Antibiotic resistance, shigellosis, Pakistan

INTRODUCTION

Shigellosis is problem in both developed and under developed countries. This is one of the most prevalent and important diseases in the developing countries (Arora *et al.*, 1982; Albert *et al.*, 1990). Shigellosis is one of the major causes of morbidity and mortality among children less than 5 years of age in developing countries. Shigellosis is usually transmitted from person to person in households (Wilson *et al.*, 1981; Makintubee *et al.*, 1987), however outbreaks due to contaminated food (Weissman *et al.*, 1974; Black *et al.*, 1978) or water (Baine *et al.*, 1975; Makintubee *et al.*, 1987) are not unusual.

Resistance of Shigellae to antibacterial drugs has been reported from different parts of the world and is increasing (Gedebou and Tassew, 1982). Resistant *S. flexneri* was first reported in Japan (Suzuki *et al.*, 1986). The development of transferable multi-drug resistance was soon reported from various parts of the world (Wang *et al.*, 1976; Chugh *et al.*, 1985; Panigrahi *et al.*, 1987; Lin and Chang, 1992; Ling *et al.*, 1993; Bratoeva and John, 1994; Casalino *et al.*, 1994; Ries *et al.*, 1994; Samonis *et al.*, 1994).

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In Pakistan, like other developing countries, there is also a general increase in drug-resistance especially to all commonly used antibiotics. Furthermore, a great worldwide proportion of Shigellae strains of various serotypes (especially, *S. dysenteriae* type I and *S. flexneri*) are now multiple drug resistant and new, simple and effective treatments of Shigellosis have not yet been developed (WHO, 1990; Hale, 1991).

In the present study the screening for antibiotic resistance was performed to study plasmid associated nature of these resistances. A number of R-plasmids were isolated during these studies and will be reported elsewhere.

MATERIALS AND METHODS

Bacterial isolates Shigellae strains were isolated from stools of patients suffering from diarrhoea or related symptoms at National Institute of Health, Islamabad, Armed Forces Institute of Pathology, Rawalpindi, and Shaikh Zayed Hospital, Lahore, Pakistan. The isolates were identified on the basis of routine biochemical and serological tests. The identification numbers used in this study are our own. Bacterial cultures were maintained in freezing glycerol LB media at -20 °C. For routine experiments, the cultures were maintained on LB agar plates at 4 °C and subcultured bimonthly.

Chemicals and media

Chemicals and antibiotics used in this study were obtained from Sigma Chemicals Co., and were of molecular biology grade. Culture media were purchased from Difco Laboratories. Difco (USA) LB broth and agar were used for the screening of cultures for antibiotic resistance.

Antibiotics used in these studies were, Ampicillin (A), Chloramphenicol (C), Enoxacin (E), Gentamycin (G), Septran (S), Tetracycline (T), Urixin (U) and Velosef (V). Stock solutions (10 mg/ml) of antibiotics were made in distilled water. Chloramphenicol was dissolved in ethanol. All solutions were sterilized by millipore (0.45 mμ) filters and refrigerated.

Antibiotic resistance/sensitivity tests

Antibiotic sensitivity was performed using diffusion method of Bauer *et al.* (1966). Minimum inhibitory concentration (MIC) was determined by an agar dilution technique using lactose neutral red peptone (LNRP) as the basal medium with 2% peptone, 1.5% Difco agar, 3.5 ml/l neutral red solution and 1% lactose. Serial dilution of antibiotics *i.e.*, ranging from 500 μg/ml to 25 μg/ml were prepared in LNRP and used in the resistance tests. The MICs of the antibiotics were tested for 24 strains. A loopful (2 mm size) of 4 h bacterial culture was inoculated on different antibiotic plates. The inoculated plates were incubated for 24 h at 37 °C before recording MICs.

RESULTS

Of the 24 strains isolated, 45.8% isolates were *S. flexneri*, 33.3% *S. boydii*, 12.5% *S. sonnei* and 8.3% *S. dysenteriae*. The antibiotic resistance was screened against these Shigellae isolates. Overall 69% of strains were resistant to Urixin (U), 68% to Septran (S), 60% to Velosef (V), 58% to Ampicillin (A), 50% to Tetracycline (T), 36% to Chloramphenicol (C), 34% to Gentamycin (G) and 29% to Enoxacin (E).

The MICs of 8 antibiotics against 24 strains of Shigellae are shown in a comparative account of the antibiotic resistance of cultures, at different levels in Fig.1. Generally, the cultures, showed the highest frequency of resistance against Septran at 50 and 100 μg/ml whereas at 250 and 500 μg/ml, the highest frequency of resistance was against Urixin. The lowest frequency of resistance was against Enoxacin at all the levels of antibiotics screened, followed by Chloramphenicol and Gentamycin. There was a slight decrease in the number of Septran resistant cultures at the levels of 50-500 μg/ml compared with the level of 25 μg/ml. At 250 μg/ml level, the cultures showed a considerable decrease in the resistance frequency of almost all antibiotics.

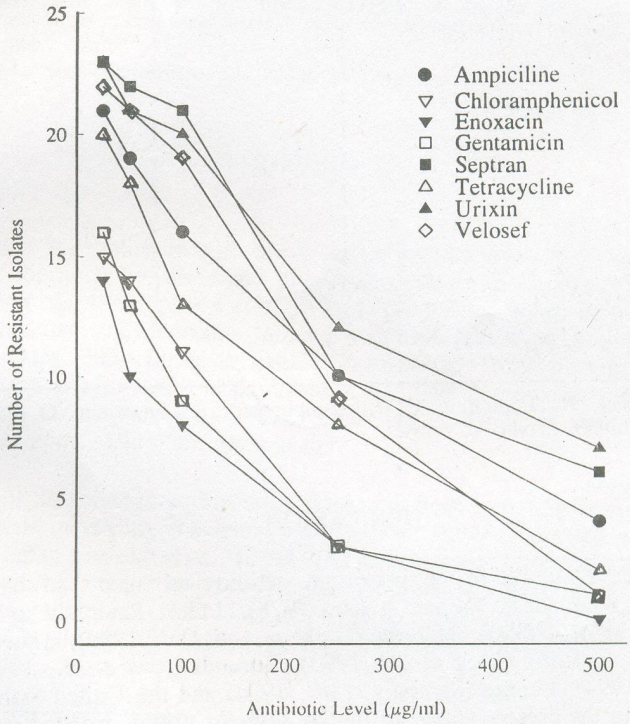

Fig. 1: Antibiotic resistance of bacterial isolates at different levels of antibiotic.

Multiple antibiotic resistance among Shigellae isolated from clinical sources is not uncommon. Out of the 24 strains screened for resistance 60.9% were resistant to one or more antibiotics at 100 μg/ml, 30.2% were resistant to one or more antibiotics at 250 μg/ml and 11.4% were resistant to one or more antibiotics at 500 μg/ml. The resistant cultures showed different patterns of antibiotics resistance at three levels of antibiotics (100 μg/ml, 250 μg/ml and 500 μg/ml) (Table I). The most common pattern was ASUV at 100 μg/ml.

Table 1: Antibiotic resistance pattern of Shigellae isolated from various clinical sources.

Resistance pattern*	Number of isolates at		
	100 μg/ml	250 μg/ml	500 μg/ml
ACEGSTUV	1	-	-
AEGSTUV	1	1	-
ACGSTU	1	-	-
ACESTU	1	1	-
AEGSUV	1	-	1
CESTUV	1	-	-
CGSTUV	1	-	-
CEGSUV	1	1	1
CGTUV	1	1	-
ACEUV	1	1	-
ASTUV	1	-	1
AGSUV	1	2	-
ASTU	2	1	1
ASUV	6	3	2
CSTV	3	2	1

* Abbreviations used: A, ampicillin; C, chloramphenicol; E, enoxacin; G, gentamycin; S, septran; T, tetracyclin; U, urixin; V, velosef.

DISCUSSION

The Shigellae continue to be important aetiological agents of dysentery and gastroenteritis in Bangladesh (Nigar *et al.*, 1978), India (Panigrahi *et al.*, 1987), Australia (Albert *et al.*, 1990), Nigeria (Olukoya and Oni, 1990), Taiwan (Lin and Chang, 1992), Hong Kong (Ling *et al.*, 1993), Burundi (Ries *et al.*, 1994), Somalia (Casalino *et al.*, 1994), Greece (Samonis *et al.*, 1994) and the United States (Bratoeva and John, 1994). In the present study, of the 24 *Shigella* strains, isolated from different Pakistani clinical sources, *S. flexeneri* is the most commonly found species (45.8%) and is followed by *S. boydii* (33.3%), *S. sonnei* (12.5%) and *S. dysenteriae* (8.3%).

The results indicate that antibiotic resistance among indigenous clinical Shigellae is very common. Out of a total 24 isolates, 23 were found to be resistant to single or multiple antibiotics. Different resistance patterns were observed among the resistant isolates. Resistance to Septran was shown to be the most common. Maximum number of the resistant isolates were found at the level of 25 μg/ml. Resistance to Enoxacin was found to be the lowest at all levels (25-500 μg/ml), whereas Gentamicin and Chloramphenicol are more effective at higher concentrations (100-500 μg/ml). Velosef and Tetracycline are effective only at 500 μg/ml. Analogous results have been reported by Shang-Yuan *et al.* (1992).

Maximum strains (69%) were found to be resistant to Urixin. This was followed by Septran (68%), Velosef (60%), Ampicillin (58%), Tetracyclin (50%), Chloramphenicol (36%), Gentamicin (34%) and Enoxacin (29%). These findings were similar to those reported from India and Taiwan, especially in case of Tetracycline and Ampicillin (Arora *et al.*, 1982; Lin and Chang, 1992) and may reflect the widespread overuse of these antibiotics especially, Tetracycline.

Multi-drug resistant Shigellae has been reported to be increasing in incidence in many countries by several workers. Kaliyugaperumal *et al.* (1978) reported multi-drug resistant in 79.4% of strains while Panikar *et al.* (1978), Arora *et al.* (1982) and Panigrahi *et al.* (1987) reported their number to be 94%, 76% and 81%, respectively. In the present study, 95.8% of the Shigellae isolates were multi-drug resistant strains. Only 4.1% of isolates were sensitive to all eight antibiotics tested. We also observed very high resistance levels in 23 strains for which MICs of the antibiotics were tested.

The prevalence of drug resistance in Shigellae observed here and elsewhere in the world indicates the futility of routine antibiotic therapy in bacillary dysentery, which is a self-limiting disease in majority of cases. Our results emphasize the urgent need to exercise a restraint in the use of oral antibiotics in Shigellosis. Samonis *et al.* (1994) also supported the view that Shigellosis can be controlled without the use of antibiotics. The overall incidence of *Shigella* infections can be decreased by improvements in the levels of environmental and personal hygiene.

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INTERACTION OF CIRCULATORY GLUCOSE AND FREE FATTY ACIDS IN INDUCED HYPERINSULINEMIC STATE IN DWARF GOATS *

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Abstract.- Single and repeated 3U/kg body weight long acting protamine zinc bovine insulin was administered to investigate the hormone's action on circulatory carbohydrate and lipid targets. High dose insulin caused marked ($P < 0.001$) hypoglycemia, which persistedbeyond ten hours after the treatment. Goats remained stable for nearly six hours under acute hypoglycemia but thereafter most of them experienced insulin shock, however, dextrose (20%) infusion overcame the shock in the goats. Low glycemia is the likely cause of enhanced FFAs levels as dextrose infusion in insulin shock promptly reduced the enhanced plasma FFA. Inspite of insulin resistance and alternative energy utilization of FFA, glucose is indispensable in ruminants. Strong interactions of carbohydrate and lipid targets exist in ruminants in hormonal homeostasis.

Key words: Insulin, glucose, free fatty acids, dwarf goat.

INTRODUCTION

Insulin is the most important regulator for glucose disposal and production. It affects glucose output and regulates the steady-state glucose level by altering the sensitivity of this control system (Jenkins *et al.*, 1986). Insulin inhibits glucose production with increased metabolic rate and its disposal from body (Moxley *et al.*, 1990). Ruminants demonstrate marked insulin insensitivity and hypoglycemic irresponsiveness (Kaneko, 1980; Cheema *et al.*, 1988) unlike non ruminants where insulin brings hypoglycemia with smaller doses and hepatic glycogenolysis restores glycemia. The increment in plasma insulin causing 50% reduction in glucose production is 50-60 $\mu U\ ml^{-1}$ (Brockman, 1983; Weekes *et al.*, 1983) whereas the comparable value for human is 30 $\mu U\ ml^{-1}$ (Rizza *et al.*, 1981). In ruminants hypoglycemia induced by high doses of insulin fails to return to the fasting level in two hours, which is the demonstration of hypoglycemia non-responsiveness (Cheema *et al.*, 1988) and the oral hypoglycemic agents failed to show the same degree of effectivity in regulating glucose via enhancing insulin release from β cells (Cheema *et al.*, 1989). Excess insulin may bring coma or convulsion in dogs, young ruminants and man but not the birds or mature ruminants (Hsu and Crump, 1989). Therefore insulin action on glycemia in ruminants are yet to be understood in various aspects.

Free fatty acids (FFAs) are the next target after glucose, to be most affected by insulin. Insulin is a principal antilipolytic hormone *in vivo* (Skarda and Bartos, 1969) and *in vitro* (Cochrane and Rogers, 1990). It plays distinct role in maintenance of lipogenic activity (Etherton and Evock, 1986) by increasing the rate of fatty acid synthesis in adipose tissue (de-la-Hoz and Vernon, 1993). Insulin regulates FFA inhibition of lipolysis while maintaining a constant rate of primary FFA reesterification (Campbell *et al.*, 1992). In ruminants particularly in sheep there are reports of insulin

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effects on lipid metabolism showing its antilipolytic effects (Cochrane and Rogers, 1990) and of a little effect of the hormone on fatty acid synthesis (Broad *et al.*, 1983).

It is generally agreed that glucose and lipid in metabolism are strongly correlated and this aspect is little understood in ruminants. The present study is carried out to investigate insulin action in ruminants, employing high dose/s, on glycemic and free fatty acid targets and to add information on the interaction of carbohydrate and lipid metabolism in hormonal homeostasis of ruminants based on dwarf goat model.

MATERIALS AND METHODS

Goat facility at Bio-Saline Research Sub-Station (BSRS) of Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad of Pakistan Atomic Energy Commission (PAEC) was used for the study. Adult billy goats of about 2 years of old with a mean body weight of 23-29kg were used. Goats kept at a farm were shifted to the animal house and acclimatized to a feeding regime. The goats were provided with chopped green fodder from 8 am to 5 pm and dry ration for a rest of the period of the day to feed *ad libitum*. Dry ration contained composition: soya bean meal 25%, wheat bran 30%, rice polishing 27.5%, molasses 15%, urea 1%, salt 0.5% and dicalcium phosphate 1%.

Two experiments were designed for *in vivo* studies using protamine zinc bovine insulin preparation in vials (Eli Lilly Italia S.p.A. Sosto Fiorentino Firenze; Italy) in concentration of 40 U/ml for injections. In single high dose experiment goats received only one injection (intravenous) of 3.0U/kg insulin. In repeated high dose experiment, goats received an injection (3.0U/kg) again every 24 hour for three days, however, on fourth day they received glucose load (0.25g/kg body weight)) soon after insulin injection. Infusions and blood sample protocol is presented in Table 1. Serum and plasma sample obtained were stored at -20^{0}C till used for analysis.

Table 1. Bovine insulin administrations and their sampling schedule

Experiment	Hours in relation to administrations from 7:00am
Single dose (i.v.) 3.0 U/kg	-15, -1, 0↓, 0.25, 0.5, 1, 2, 4, 7S, 7.2R, 9.75S, 10R, 24
Repeated dose 3.0 U/kg (i.v.)	-15, -1, 0↓, 0.5, 6, 24↓, 24.5, 30S, 31R, 48↓, 48.5, 52S↑, 52.5R, 56S↑, 56.5R, 72↓↑, 72.25, 72.5, 73, 75, 76↑, 79, 96, 102

Insulin injection: ↓, Shock: S, Recovery: R, & Combined administration of insulin and glucose: ↓↑

Blood glucose was estimated by oxidase method (Barham and Trinder, 1972) and commercial kits (Randox Laboratories Ltd., Ardmore, U.K) were used. The method of Falholt *et al.* (1973) using copper soap formation was used for total plasma free fatty acids (FFAs). The significance of differences among the different experimental steps following administrations etc. were analyzed by one way analysis of variance (ANOVA). When F -test was significant ($P < 0.05$), contrast of the steps were tested with least significance difference [LSD] (Sokal and Rohlf, 1981).

RESULTS

FEED CONSUMPTION

Significantly reduced feed consumption during high dose insulin treatment is most likely due to the reason that goats remained uncomfortable under insulin influence. There is indication, however, of insulin role in appetite as consumption increased in days following a single dose experiment and reduction of consumption was marked when insulin was administered along dextrose.

Table 2. Total feed consumption per goat per day (dry weight in g)

Experimental state	Day 1	Day 2	Day 3	Day 4
Control phase	1102 ± 75,	789 ± 44,	1148 ± 19,	1007 ± 36
Single dose treatment phase	627 ± 17			
Post-treatment phase	1188 ± 80,	1204 ± 81,	1110 ± 103	
Repeated doses treatment phase	1004 ± 17,	707 ± 142,	852 ± 149	
Insulin + Dextrose treatment phase	418 ± 161			

GLUCOSE

Single dose: Prior to hormone administration the average concentration of plasma glucose was 60.70 ± 1.2 mg/dl. Maximum reduction of 65% ($P < 0.001$) in glycemia had occurred half an hour post-treatment, it persisted in a narrow range of fluctuations upto seven hours post-treatment, when two of the goats suffered shock, with glycemic levels 15 & 18 mg/dl. Twenty minutes after intravenous glucose infusion to these goats,

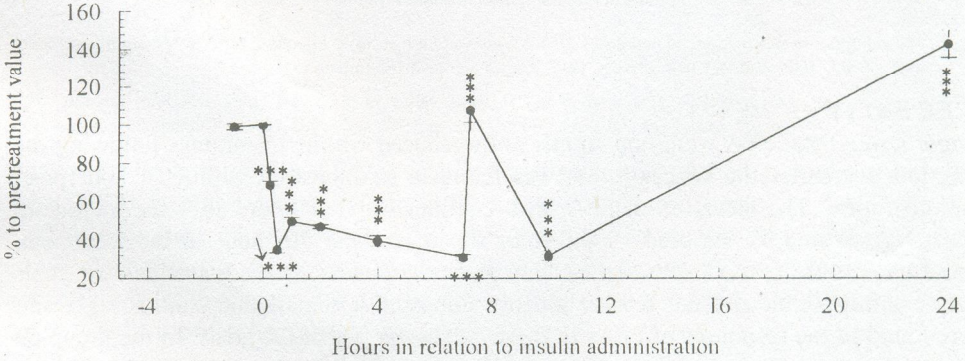

Fig. 1. Plasma glucose following single dose 3U/kg B.W. insulin. ↓ injection, *** significant level at $P < 0.001$.

glycemic level was in the range of pretreatment level. Ten hours after again hypoglycemic state returned with another shock to the same goats. Another glucose infusion kept the goats stabilized up to next morning. Twenty-four hours after glycemic

level was significantly ($P < 0.001$) greater than pre-treatment control values a day earlier (Fig. 1).

Repeated dose: Just before hormonal administration the average glycemic level was 54.05 ± 1.4 mg/dl in male dwarf goats. Marked hypoglycemia of 62% ($P < 0.001$) half an hour after injection and its persistence ($P < 0.001$) upto six hour resembled its short term pattern. All the goats remained stable during first day. With the advancing days, six hours post treatment the level was comparatively lower than the earlier day respective value, and three goats suffered insulin shock with plasma glucose 27, 19 & 23 mg/dl, thus it required glucose therapy. On the third day, one goat received fatal shock. The shock was so severe and unrecoverable that even after emergency treatments of glucose, goat died 1.75 hours after insulin injection with glycemic level 9 mg/dl. The rest of the goats received shock six hours after insulin injection from which they recovered after glucose therapy. On fourth day three hours post-combined treatment of insulin and glucose samples showed presence of severe hypoglycemia ($P < 0.001$) with the reduction of 78%; however, all the goats remained stable (Fig. 2).

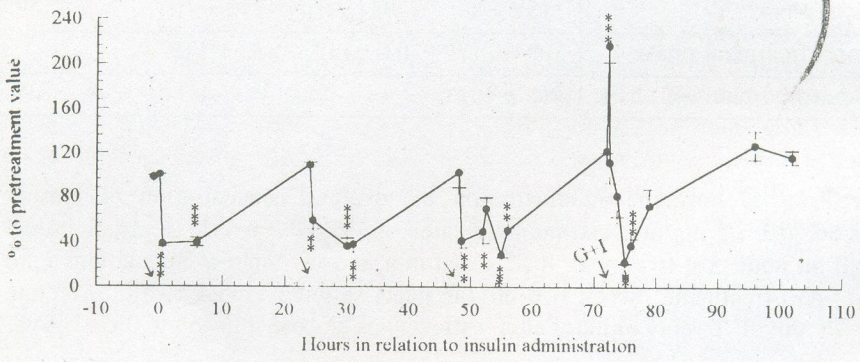

Fig. 2. Plasma glucose following repeated doses 3U/kg body weight insulin. ↓ injection, *, ** & *** significant level at $P < 0.05$, 0.01 & 0.001 respectively, G+I, insulin and dextrose infusion.

FREE FATTY ACIDS

Single dose: Total FFA were non significantly reduced within 15 minutes following the injection, thereafter the concentration was found to be increased within an hour post-administration. The elevation in FFA level continued upto 7 hours after the treatment. Goats No. 74 and 75 suffered with insulin shock at about 7th hour of the treatment. These goats were treated with intravenous glucose infusion; while the other two goats that remained stable did not receive glucose infusion. A remarkable contrasting result were found in the responses of FFAs in these two categories of the goats. In the group on glucose therapy, elevated plasma FFAs level were markedly lowered within 20 minutes after dextrose infusion. In other group which did not receive dextrose, FFA levels remained elevated. About 3 hours after the shock and glucose therapy, the lowered FFA level again increased in goats No. 74 and 75. In all the goats 24 hours after insulin treatment FFA level was found elevated than pre-treatment control values (Fig. 3).

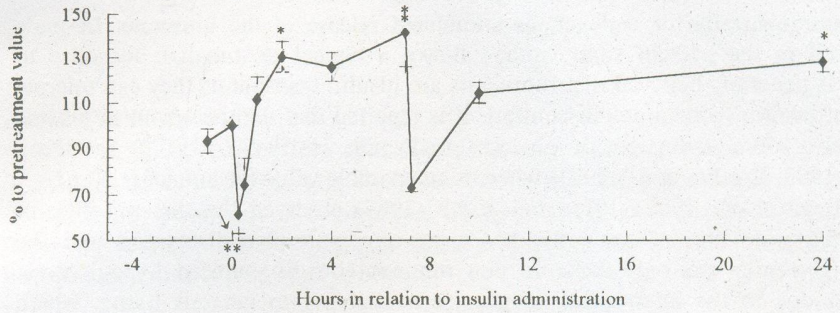

Fig. 3. Plasma free fatty acids following single dose 3U/kg body weight insulin. ↓ injection, * & ** significant level at P < 0.05, & 0.01 respectively.

Repeated Dose: The pattern in the long term treatment with high doses further confirmed the FFAs responses as found in short term high dose experiment. Within half an hour FFAs were markedly lowered (P 0.001), which later were found elevated even compare to an average control value at 6-7 hours post-treatment. The same pattern was found on second day. All the goats except 86 had suffered shock, so were given dextrose

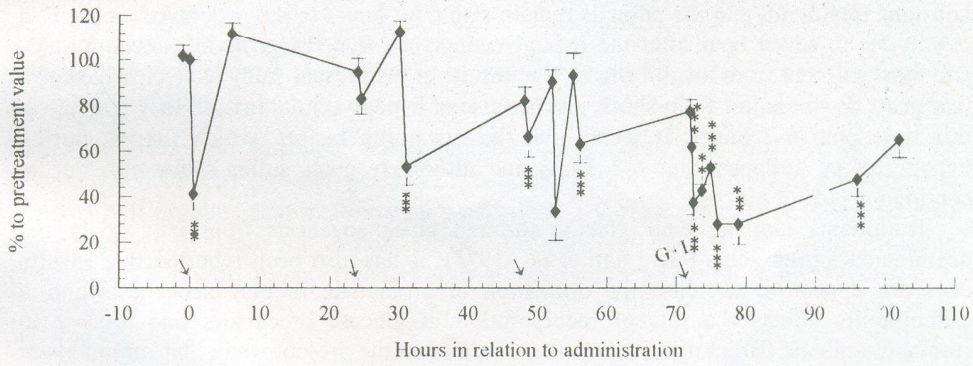

Fig. 4. Plasma free fatty acids following repeated doses 3U/kg body weight insulin. ↓ injection, *, ** & *** significant level at P < 0.05, 0.01 & 0.001 respectively, G+I, insulin and glucose infusion.

infusion. In these three goats FFAs were lowered soon after glucose administration. In goat 86 FFAs level did not decrease as it did not receive glucose therapy.

The combined administration of insulin and glucose on 4th day, confirmed the results of the earlier days that in the presence of glucose FFAs remain lower in concentration following insulin administration even upto 24 hours. Pretreatment level was restored within 30 hours after the injection (Fig. 4).

DISCUSSION

The major function of insulin is to facilitate the transport or flux of glucose across the plasma membrane of cells of most tissues, thus hypoglycemia ensues following

exogenous administration or endogenous stimulated release of the hormone. In male goats, as used in the present study, it has shown a typical mammalian response to glycemia. It is generally believed that ruminants are insulin resistant as they can tolerate high doses of insulin without any discomfort. It is reported that the increment in plasma insulin causing 50% reduction in glucose production is 50-60 μU^{ml-1} in sheep (Brockman, 1983; Weekes *et al.*, 1983) whereas comparable value for human is 30 μU^{ml-1} in man (Rizza *et al.*, 1981). Prior and Smith (1983) observed that the injection of insulin (6U/kg) into normal cattle did not bring any noticeable discomfort and the decrease in glycemia was only 22%. In non ruminant insulin induced hypoglycemic shock results due to the interruption of glucose availability to nervous tissue, which otherwise cannot store energy for emergency conditions. In ruminants, on the other hand, a fewer availability of glucose is conventionally compensated by fatty acid fractions (Preston and Leng, 1987). Therefore, ruminants have adapted to manage with very low levels of glycemia.

The long acting zinc protamine insulin, compared to regular, persists for longer duration in the circulation and can maintain hypoglycemia for several hours. In the goat, used as a model for ruminants, present study has shown that high doses of long acting insulin (3U/kg body weight) induce marked hypoglycemia within an hour after the hormone treatment, and the animals remain stable for hours in the hypoglycemic state. It is only six to seven hour after the hypoglycemic state that the goats felt uncomfortable and most suffered from insulin shock. The results of the present study have clearly shown that goats do not suffer from shock just because of hypoglycemia, instead they manage in this state upto 6-7 hours. It seems that the sustaining factors, which support during hypoglycemia, collapse after 6-7 hours and ultimately goats suffer shock also due to continuous lack of glucose.

Ruminants manage their glucose through gluconeogenesis from fatty acids and deaminated amino acids (Bergman *et al.*, 1974). It has also been reported that insulin does not appear to decrease the utilization of propionate in gluconeogenesis and it differentially affects the rate of incorporation of glucose precursors into glucose in ruminant animals (Brockman, 1990). It is revealed in the present study that ruminants or at least the dwarf goat can manage in consistent low glycemic level while using lipid constituents already present in the cells. It is also observed that the prolonged hypoglycemic condition, due to high insulin level, consequently affects adversely and inhibits the further transport of fatty acids into the cells. As the cellular energy stores are completely depleted the prolongation of the starvation of the cells for energy constituents eventually brings insulin shock even in the goat. It is clearly demonstrated in the results of present study that with the prolongation of hypoglycemia in high dose insulin treatments, plasma free fatty acids levels increased because of their inability to be transported into the cells. The reason for the lack of transport of FFA into the cells is clearly due to a low circulatory glucose, as following exogenous glucose therapy, as the level of glycemia rises, circulatory free fatty acids are declined due to their transportation into the cells. In the present study glycemic level did not reach to zero level, thus a question arises of the inability of the already available, although a low, glucose role in the circulation. This may have been due to elevated FFA as Ferrannini *et al.* (1983) and

Gomez *et al.* (1972) have observed that increased plasma FFA decrease insulin- mediated glucose uptake, glucose oxidation and glucose storage. Fetal plasma FFA increased significantly during hypoglycemia (Harwell, 1990) and also plasma FFA and urea rose in response to fasting in rumiants (Robinson *et al.*, 1992; Ward *et al.*, 1992). The results of the present study have clearly demonstrated that free fatty acids transport is adequately bound with sufficient level of circulatory glucose. There have been investigations to understand the kinetics of nutrients transport across the cell membrane. Hansen *et al.* (1992) have shown that insulin responsiveness to glucose is enhanced by simultaneous insulin exposure. Thus the transport of circulatory nutrients into the cell is just not between a single nutrient and its transporting hormone but more complex in the facilitation and inhibition rendered to one another in metabolites in relation to the hormone. It has been observed that insulin infusion into sheep portal circulation depressed glucose entry immediately (West and Passey, 1967). Hypoglycemia secondary to insulin infusion, imposes a significant stress on the organism and triggers the release of various counterregulatory hormones that tend to affect the action of insulin on glucose metabolism (Frizzell *et al.*, 1988; Gerich *et al.*, 1979). It is associated with enhanced hepatic glucose production, lipolysis, and ketogenesis (Frizzell *et al.*, 1988). Insulin is understood to increase the rate of fatty synthesis in adipose tissue (de-la-Hoz and Vernon, 1993).

It is evident, from the study, that inspite of insulin resistance and even in alternate source of energy of FFA, in intense hypoglycemia, due to prolonged insulin action, glucose is still indispensable for, at least, free fatty acid transport into the tissues of ruminants. Also an intense carbohydrate and lipid metabolic interaction is espied in these animals.

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