STUDIES ON TOXICOPATHOLOGY OF SODIUM BENZOATE IN RATS

M.V. Sc. THESIS

BY

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DEPARTMENT OF VETERINARY PATHOLOGY

COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY

ANJORA, DURG

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THESIS

Submitted to the

INDIRA GANDHI KRISHI VISHWAVIDYALAYA, RAIPUR

BY

DEEPMALA DEWANGAN

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OF

Master of Veterinary Science

In

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CERTIFICATE – I

This is to certify that the thesis entitled **"Studies on toxicopathology of sodium benzoate in rats"** submitted in partial fulfillment of the requirements for the degree of **Master of Veterinary Science** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.), is a record of the bonafide research work carried out by **Deepmala Dewangan** under my guidance and supervision. The subject of the thesis has been approved by the student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma or has been published/published part has been fully acknowledged. All the assistance and help received during the course of investigations have been duly acknowledged by her.

Date: **Chairman**

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Member:	**Dr. Mohan Singh** Professor and Head	**……...**
Member:	**Dr. K. M. Koley** Professor and Head	**……...**
Member:	**Dr. S. K. Maiti** Professor and Head	**……...**
Member:	**Dr. M. Mondal** Assistant Professor	**……...**

CERTIFICATE – II

This is to certify that the thesis entitled **"Studies on toxicopathology of sodium benzoate in rats"** submitted by **Deepmala Dewangan** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.) in partial fulfillment of the requirements for the Degree of **Master of Veterinary Science** in the Department of Veterinary Pathology has been approved by the student's Advisory Committee after oral examination in collaboration with the External Examiner.

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VITA

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Date: ***DEEPMALA DEWANGAN***

Anjora, Durg

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LIST OF SYMBOLS/ABBREVIATIONS

Abbreviation	Full Form
%	per cent
@	at the rate of
µ	micron
µg	microgram
µl	microliter
^{0}C	degree Celsius
^{0}F	degree Fahrenheit
AFCO	animal food corporation organization
ALD	approximate lethal dose
ALP	alkaline phosphatase
ALT	alanine amino transferase
AST	aspartate amino transferase
BUN	blood urea nitrogen
b.wt	body weight
CDER	center for drug evaluation and research
CMI	cell mediated immunity
DCT	distal convoluted tubules
dl	deci liter
DLC	differential leucocyte count
DNFB	dinitrofluorobenzene
FDA	food and drug administration
fl	femtolitre
g	gram
Gr	group
h	hour(s)

H&E	haematoxylin and eosin
HA	haemagglutinin
Hb	haemoglobin
i.e.	that is
IU	International Unit
kg	kilogram
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
mg	milligram
MNCs	mono nuclear cells
NOAEL	no observable adverse effect level
NSS	normal saline solution
PBS	phosphate buffer saline
PCT	proximal convoluted tubules
PCV	packed cell volume
Pg	pico gram
ppm	parts per million
rpm	rotation per minute
SRBC	sheep red blood cells
Tab	tablet
TEC	total erythrocyte count
TLC	total leucocyte count
viz.	namely
WBC	white blood cells

Chapter-I

Introduction

As is said that except the food grown in your own garden, all food products have preservatives. Because food is so important to survival, food preservation is one of the oldest technologies used by human beings. The deterioration of some foods caused by the activity of microorganisms may be prevented and the keeping quality of the foods greatly improved by the addition of certain chemical compounds. It is obvious that preservatives which either produce a marked toxic effect or the consumption of food containing unusual quantities of preservatives may be dangerous and harmful for both human as well as animal's consumption because of the hidden cumulative effect.

Artificial preservatives are a group of chemical substances added to food, sprayed on the outside of food, or added to certain medications to retard spoilage, discoloration, or contamination by bacteria and other disease organisms. Sugar and salt are often used as preservatives. Some modern synthetic preservatives have become controversial because they have been shown to cause respiratory or other health problems (Bateman *et al*., 2004). One of such chemicals known as sodium benzoate has been taken into consideration as toxicological studies are concerned.

The sodium salt of benzoic acid is used as an antifungal preservative in pharmaceutical preparations and foods. Sodium benzoate (NaB, C_6H_5COONa), EU No. (E 211), molecular weight 144.11, also called benzoate of soda, is a polyunsaturated fat. Sodium benzoate (CAS No. 532-32-1) is about 200 times

more soluble in water than benzoic acid. Normally used concentrations are 0.05 to 0.1 % in fruit juice and 0.1 % in skin creams. Sodium benzoate was the first chemical preservative permitted in food for human consumption in the U.S. in 1908 and continues to be used in a large number of foods (Jay, 1992). The international program on chemical safety found no adverse effects in humans at doses of 647-825 mg/kg body weight per day (Nair, 2001).

Since the early 1900's, sodium benzoate has been used as a food preservative. Worldwide sodium benzoate production in 1997 was estimated at about 55000-60000 tonnes (Srour, 1998). Sodium benzoate is utilized in a wide range of products we consume through everyday use such as beverages, fruit juices, syrups, olives, pickles, tooth pastes, mouth washes, dentifrices, cosmetics, pharmaceuticals (Srour, 1998) and other condiments as antimicrobial preservative due to its low toxicity and low taste. In animal feeds the use of benzoic acid has increased during recent years. Benzoate inhibits fungal growth in formic acid treated grass silages (Aronen *et al*., 1987). It is also used as anticorrosive in automotive engine (Scholz and Kortmann, 1991 and Srour, 1998) and in plastics such as polypropylene, to improve strength and clarity (Kalama, 1999). Benzoic acid and sodium benzoate are generally recognized as safe in foods according to the U.S. Food and Drug Administration (Nair, 2001). But some reports suggest adverse effects due to both chronic and sub-chronic intake of sodium benzoate (Fujitani, 1993 and Vogt *et al*., 1999).

Sodium benzoate is a white, crystalline solid and is a hygroscopic material. It is easily dissolved in water, forming a transparent, colorless solution. Sodium benzoate is, however, allowed as an animal food additive at upto 0.1%, according

to AFCO's official publication (AFCO, 2004). It has both fungistatic and bacteriostatic activity. The liver is the main site of conjugation with glycine in both man and most experimental animals (rabbits and rats). Cumulation does not occur, as shown by experiments, on the distribution and elimination of sodium benzoate-1-C^{14} administered intraperitoneally, orally or sub cutaneously to the rat. Benzoic acid can cause skin and eye irritation. Sodium benzoate only causes eye irritation however it is an active ingredient in many oral medications (Heather, 2007). Sodium benzoate in food and drink products appears to form a chemical known as benzene in the presence of vitamin C. Benzene not only causes damage to DNA, the genetic material, it's also a known carcinogen and appears to play a role in a variety of diseases due to its DNA damaging capabilities (Kristie Leong, 2009).

Like other aromatic carboxylic acids, benzoic acid is xenobiotic and is detoxified and eliminated from the body, mainly by conjugation with glycine (Tanaka and Isselbacher, 1967; Hutt and Caldwell, 1990 and Tremblay and Qureshi, 1993) and to a lesser extent with glucuronic acid (Bridges *et al*., 1970). After oral and dermal uptake, benzoate and benzoic acid are rapidly absorbed from the gastrointestinal tract in experimental animals or humans (US FDA, 1972 and 1973) and is metabolized in the liver by conjugation with glycine, resulting in the formation of hippuric acid (Feldmann and Maibach, 1970; US FDA, 1972; WHO, 1996 and Feillet and Leonard, 1998) and benzoyl-glucuronic acid in dog (Bridges *et al*., 1970) which rapidly excreted in urine. The limiting factor in the biosynthesis of hippuric acid is the availability of glycine for detoxification of benzoate resulting in reduction of glycine level of the body. Therefore, the

ingestion of benzoic acid or its salts affects any function or metabolic process in which glycine is involved. It leads to a reduction in creatinine, glutamine, urea and uric acid levels (US FDA, 1972, 1973, Kubota and Ishizaki, 1991 and WHO, 1996). As an exception to all other studied species, the cat (family *Felidae*) is rather sensitive to benzoic acid, due to an inability to produce benzoyl glucuronide (Bedford and Clarke, 1972). Potassium deficiency also occurs as shown by the usual symptoms of severe muscular weakness and tremors (Martin, 1966). In the blood, benzoates exist in the free state and are not bound to proteins (Knoefel and Huang, 1956).

It appears that very little work has been done on toxicopathological and immunological aspect of sodium benzoate. Therefore, in the light of the above, the present investigations were carried out with the following objectives:

1. **To study the acute toxicity of sodium benzoate in albino rats**
2. **To evaluate the hematological and biochemical alterations in the sub acute sodium benzoate toxicity**
3. **To assess the status of cell mediated and humoral immune response during sub acute sodium benzoate toxicity**
4. **To study the gross and histopathological changes in various organs by sodium benzoate**

Chapter-II

Review of Literature

2.1 PRESERVATIVE

Ogiehor and Ikenebomeh (2004) investigated the antimicrobial effects of sodium benzoate on growth, survival and aflatoxin production potential of some species of *Aspergillus* in garri during storage and observed a decrease in viable count of *Aspergillus* with no aflatoxin in samples treated with different concentrations of sodium benzoate.

2.2 METABOLISM

Mitch and Brucilow (1982) administered sodium benzoate in human patients with chronic renal failure @ 10g/ day and recorded increased hippurate nitrogen excretion per day equivalent to 70±5% sodium benzoate leading to decrease in serum urea nitrogen concentration. But there was no significant decrease in plasma concentration of glycine and serine.

Bridges *et al*. (1970) examined the urinary excretion of orally administered [14C] benzoic acid in man, rhesus monkey and rodents @ 1, 20 and 50 mg/kg body weight respectively and observed that benzoic acid was excreted entirely as hippuric acid.

Bedford and Clarke (1972) induced benzoic acid toxicity in cats by administering benzoic acid @ 450-890 mg/kg body weight in diet and observed that the sensitivity of the cat may be due to its failure to form benzoyl glucoronide.

2.3 GENERAL USE

Ishida (1996) studied the daily intake of preservatives *i.e.* benzoic acid and PHBA- ethyl @ 4450 and 1550 mg/kg respectively from commercial tooth pastes in 40 female students during brushing of teeth and reported that the daily intake of benzoic acid was about 2.23 mg, which was almost same as that from the diet.

Parfitt *et al.* (1999) and Kibbe and Arthur (2000) advocated that sodium benzoate is primarily a preservative and corrosion inhibitor with the permitted levels in oral and parentral medicines as upto 0.5%.

Davis *et al*. (2001) explored the effects of sodium benzoate and fluoride in combination and alone on dental caries in intact and desalivated rats as animal model and revealed that a combination of benzoate and fluoride reduced caries activity more effectively in rodents fed a carcinogenic diet *ad libitum* than fluoride alone.

FDA (2001) reported that benzoic acid and sodium benzoate were used as antimicrobial agents, flavouring agents and as adjuvants with a current maximum level of 0.1% in food.

2.4 MAMMALIAN TOXICITY

Bio-Fax (1973) reported the oral LD_{50} values (administration by gavage) of benzoic acid in rats are 3040 mg/kg body weight and suggested that the acute toxicity of benzoic acid is low. McCormick (1974) and Abe *et al.* (1984) found the oral LD_{50} values of benzoic acid in mice were 1940-2263 mg/kg body weight. Smyth and Carpenter (1948), Duel *et al*. (1954) and Bayer (1978) observed the oral LD_{50} values

of 2100-4070 mg sodium benzoate/kg body weight in rats and revealed that the acute toxicity of sodium benzoate is similar to that of benzoic acid.

2.5 TOXICOLOGICAL PROFILE

Kieckebusch and Lang (1960) conducted a four generation experimental toxicity study in rats by oral administration of benzoic acid @ 0.25 or 0.5 g/kg body weight/day and observed no unfavourable side effects on growth, food utilization, duration of life, feeding of the off-spring, weight of organs and histopathological pattern of organs in higher dose level.

Njagi and Gopalan, (1982) induced the sodium benzoate toxicity @ 0.05 to 50000 ppm in rats and found remarkable inhibition of DNA synthesis and the induction of anaphase bridges and subsequent micronuclei of *vicia faba* root mitotic cells. *In vitro* mutagenicity studies demonstrated that sodium benzoate induced chromosomal aberrations in rat cells with positive mutagenic activity in recombination (REC) assay.

Toth (1984) induced experimental oral intoxication of sodium benzoate @ 2% solution in drinking water for life in randomly bred swiss mice and noted no detectable tumorigenic effect.

Maswoswe *et al.* (1986) demonstrated the effect of induced sodium benzoate toxicity @ 9.5 and 2.5 mmoles/kg body weight in rats and revealed that at 9.5 mmoles/kg body weight sodium benzoate sharply increased mortality in rats subsequently challenged with ammonia while at 2.5 mmoles/kg body weight, benzoate was observed to protect fasted animals against ammonia toxicity.

Oyanagi *et al*. (1987) studied cytotoxicities of sodium benzoate @ 100 and 500µg/ml) in primary culture of hepatocytes of adult rat liver by collagenase perfusion technique maintaining a monolayer in serum free culture medium and revealed suppression in activities of ornithine transcarbamylase (marker of mitochondria) and tyrosine amino transferase (marker of cytosol) in higher dose group and suppression in intra-cellular protein and DNA synthesis in lower dose group.

Stefanidou *et al*. (2003) investigated the cellular action of food additives (food color tartrazine, the preservatives sodium nitrate and sodium benzoate and the antioxidant BHT) using the protozoan tetrahymenapyriformis as a toxicological model and recorded a statistically significant increase in DNA content and stimulation of the mitotic process which substantially alter susceptibility to chemical carcinogenesis.

Williams and Lock (2005) experimentally observed nephrotoxicity in male alderly park rats pretreated with sodium benzoate @ 500 mg/kg body weight, 48 hour after D- serine administration. However, he found that prior exposure to sodium benzoate prevents the initial onset of D-serine-induced nephrotoxicity, renal injury was still apparent at later time points.

Heuy-Jen *et al*. (2007) studied the toxicity and teratogenecity of sodium benzoate at varying concentrations (1-2000 ppm) in zebrafish and reported 100% mortality of zebrafish embryos in higher dose levels *i.e.* 2000 ppm. They also reported gut abnormalities, malformation of pronephros, defective hatching glands

and edema in pericardial sac suggesting neurotoxicity and nephrotoxicity of sodium benzoate on zebrafish larvae.

Torkoglu (2007) studied the effects of food preservatives on root tips of *Allium cepa* L. by using sodium benzoate, boric acid, citric acid, potassium citrate and sodium citrate treated @ 20 to 100 ppm for 5, 10 and 20 h and noted reduced mitotic division and variations in the percentage of mitotic stages. Different abnormal mitotic figures *viz.*, anaphase bridges, c-mitosis, micronuclei, lagging, stickiness, breaks and unequal distribution were observed in all mitotic phases.

Chen *et al*. (2009) investigated the induced intoxication of sodium benzoate in zebrafish larvae and revealed significant developmental defects in motor neuron axons and neuro muscular junctions, thereby concluded that its neurotoxicity affects the development of dopaminergic neurons in the zebrafish brain.

2.6 CLINICAL SIGNS

Kieckebusch and Lang (1960) produced experimental toxicity in twenty-eight young rats by administering 5% sodium benzoate in diet for three weeks and found significant reduction in food consumption, diarrhoea and death of nineteen animals within two weeks.

Bio-Fax (1973); McCormick (1974) and Abe *et al*. (1984) noted clinical signs of intoxication including diarrhoea, muscular weakness, tremors, hypoactivity and emaciation in rats by oral administration of benzoic acid @ 3040 mg/kg body weight.

Maswoswe *et al*. (1986) demonstrated the effect of induced sodium benzoate toxicity @ 9.5 and 2.5 mmoles/kg body weight in rats and revealed that at 9.5 mmoles/kg body weight sodium benzoate sharply increased mortality in rats

subsequently challenged with ammonia while at 2.5 mmoles/kg body weight, benzoate was observed to protect fasted animals against ammonia toxicity.

2.7 BODY WEIGHT

Duel *et al.* (1954) induced experimental toxicity in rats by oral administration of sodium benzoate @ 640, 1320, 2620 and 6290 mg/kg body weight/ day in the diet for 90 days and noted that the average weight gain of surviving rats was reduced.

Kieckebusch and Lang (1960a) produced experimental sodium benzoate toxicity in 28 young rats by giving a diet containing 5% sodium benzoate for 3 weeks and recorded death with severe weight loss in five rats within five weeks. They also observed gut hemorrhages and nasal blood crust with normal urine during autopsy.

Kowalewski (1960) experimentally induced toxicity in 15 rats by giving 5% sodium benzoate and 5% benzoate + 1% glycine in their diet for three weeks and recorded reduction in body weight, significant reduction in phospholipids in liver but no changes in total cholesterol content in liver of rats at 5% level of sodium benzoate. However, 1% glycine in diet causes reduction in body weight to a lesser extent compared to 5% benzoate containing diet.

Fanelli and Halliday (1963) studied the effect of sodium benzoate on body weight in rats and found slight decrease in body weight of rats at 2200mg sodium benzoate /kg body weight and significant body weight depression @ 2.0 to 2.4g/kg b.wt sodium benzoate in the diet for 28 days in male rats. However, death was observed at 5.7g/kg b.wt for females and 7.8g/kg b.wt of sodium benzoate for males during the experiment.

Sodemoto and Enomoto (1980) found that all the rats fed 8% level and 19 rats on 4% level of sodium benzoate in the diet died with significant reduction of body weight gain.

Moreno (1989) observed that rats fed 8% dose level of sodium benzoate in the diet for 90 days showed reduction in average body weight gain and significant increase in the relative liver and kidney weight.

2.8 ORGAN WEIGHT

Duel *et al.* (1954) induced experimental toxicity in rats by oral administration of sodium benzoate @ 640, 1320, 2620 and 6290 mg/kg body weight/ day in the diet for 90 days and recorded that the relative liver and kidney weight were significantly increased.

Fujitani (1991) induced sodium benzoate toxicity in rats by oral administration of 1568 mg sodium benzoate /kg body weight per day in feed for 10 days and found increased relative liver and kidney weights and changes in serum parameters.

Fujitani (1993) produced experimental sodium benzoate toxicity in F344 rats and B6C3F1 mice of each sex by administering sodium benzoate (0, 1.81, 2.09 and 2.40% for rats and 0, 2.08, 2.50 and 3.0% for mice) in diet for 10 days and recorded significant increase in absolute liver and kidney weight in male mice of 3% group.

2.9 HEMATOLOGY

Ibekwe *et al*. (2007a) induced short term sodium benzoate toxicity in male wistar albino rats by oral administrations of varying concentrations (30, 60 and 120 mg/kg body weight) of sodium benzoate at 2 days interval for 14 days and noted the

hematological changes comprising a significant hypohemoglobulinemia and leucopenia.

2.10 BIOCHEMICAL STUDIES

2.10.1 Glucose, total protein, albumin and globulin

Fujitani (1993) produced experimental sodium benzoate toxicity in F344 rats and B6C3F1 mice of each sex by administering sodium benzoate (0, 1.81, 2.09 and 2.40% for rats and 0, 2.08, 2.50 and 3.00% for mice) in diet for 10 days and observed the biochemical changes comprising of significant increase in the levels of albumin, total protein and gamma glutamyl transpeptidase in male rats of 2.40% group and serum cholesterol and phospholipids level in male mice of 3% group.

Ibekwe *et al*. (2007) produced experimental toxicity in male albino wistar rats by giving sodium benzoate @ 30, 60 and 120 mg/kg body weight orally at 2 days interval for 14 days and observed no significant alterations in the plasma protein concentrations.

2.10.2 Alanine amino transferase, aspartate amino transferase and alkaline phosphatase

Michael *et al*. (2005) investigated *in vitro* effects of oral administration of sodium benzoate (@ 0.03 to 0.21%) on the activities of aspartate amino transferase, alanine amino transferase and alkaline phosphatase in human erythrocytes of different genotypes (HbAA, HbAS and HbSS) and found significant inhibition of the three enzymes from the erythrocytes of HbAA, HbAS and HbSS genotypes by sodium benzoate at 0.1% level (the recommended concentration used for preservative).

Ibekwe *et al*. (2007a) produced experimental toxicity in male albino wistar rats by giving sodium benzoate @ 30, 60 and 120 mg/kg body weight orally at 2 days interval for 14 days and observed significant increase in serum levels of the aspartate amino transferase and alkaline phosphatase.

2.10.3 Creatinine, blood urea nitrogen and cholestrol

Fujitani (1991) produced experimental toxicity in rats for 10 days by oral administration of sodium benzoate @ 1358 mg/kg body weight per day in feed and found increase in serum cholesterol levels of female rats.

Abd-AlGadir *et al*. (2009) induced experimental benzoic acid toxicity in rats by oral administration of benzoic acid @ 100, 500 and 1250 mg/kg body weight and recorded the biochemical alterations which consisted of significant increase in the serum creatinine and urea nitrogen levels in the rats with increasing dose of benzoic acid.

2.11 IMMUNOLOGICAL STUDIES

Sodemoto and Enomoto (1980) observed that the rats fed with 0.5 to 8% sodium benzoate in the diet for a period of six weeks show hypersensitivity as an acute toxic effect with no other symptoms.

Gad *et al*. (1986) and Gerberick *et al*. (1992) conducted a maximization test and Buehler test in 15 guinea-pigs and ear swelling test and local lymph node assay in mice after induction and challenge with 10-20% solution of benzoic acid in water and found none of the guinea pigs and mice reacted positively.

Brahmachari and Pahan (2007) revealed that sodium benzoate modifies encephalitogenic T cells at multiple steps in mice and it may have therapeutic importance in multiple sclerosis.

2.12 PATHOLOGY

Duel *et al.* (1954) induced experimental toxicity in rats by oral administration of sodium benzoate @ 640, 1320, 2620 and 6290 mg/kg body weight/ day in the diet for 90 days and observed significant histopathological changes in liver and kidney.

Kieckebusch and Lang (1960) observed gut hemorrhages and nasal blood crust in young rats, given a diet containing 5% sodium benzoate for three weeks.

Bedford and Clarke (1972) studied the histopathological effects of oral administration of 1% benzoic acid (450-890 mg/kg body weight) in cats and revealed degenerative changes in liver, kidneys and lung. However, there were no pathological findings in brain or spinal cord.

Sodemoto and Enomoto (1980) found that all the rats fed 8% level and 19 rats on 4% level of sodium benzoate in the diet died with atrophy of spleen and lymph nodes as morphological changes at autopsy.

Toth (1984a) experimentally administered benzoic acid @ 647 mg/kg body weight in diet in rats for 4 weeks and sodium benzoate @ 6200 mg/kg body weight in drinking water in mice for life long and observed no observable effects on the testes of both rats and mice.

Fujitani (1991) produced experimental toxicity in rats for 10 days by oral administration of sodium benzoate @ 1800 mg/kg body weight per day in feed and

found histopathological changes in liver and disorders of the central nervous system (convulsions).

Fujitani (1993) produced experimental sodium benzoate toxicity in F344 rats and B6C3F1 mice of each sex by administering sodium benzoate (0, 1.81, 2.09 and 2.40% for rats and 0, 2.08, 2.50 and 3.00% for mice) in diet for 10 days and found enlarged hepatocytes with glassy cytoplasm in male rats of 2.40% group and enlargement, vacuolation and necrosis of hepatocytes in male mice of 3% group.

Huey-Jen *et al*. (2007) observed misalignment of muscle fibers, motor neuron innervations, excess acetyl-choline receptor cluster and defective pronephric tubes in zebrafish larvae treated with sodium benzoate and suggested that sodium benzoate induce neurotoxicity, nephrotoxicity and malformation of zebrafish larvae.

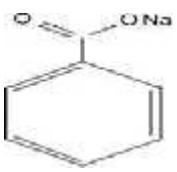
Chapter-III

Materials and Methods

The present study consists of acute and subacute oral toxicopathological evaluation of sodium benzoate. In the acute toxicity study approximate lethal dose (ALD) of sodium benzoate in albino rats was determined. The sub acute toxicity was conducted to study the effect of sodium benzoate on various systems of both male and female rats after giving the exposure for 28 days.

3.1 FOOD PRESERVATIVE:

Sodium benzoate technical grade was supplied by Qualigens®, GlaxoSmithKline Pharmaceuticals Ltd., Dr. Annie Besant Road, Mumbai, India. The purity of the sodium benzoate technical grade was 99%. Identity of the active substance and the preparation are as follows:

Active Substances:	Sodium benzoate
IUPAC Name	Sodium benzoate
Function:	Food preservative
CAS number:	532-32-1
Molecular Formula:	$C_7H_5O_2Na$
Molecular weight:	144.11
Structural Formula:	

3.2 EXPERIMENTAL ANIMALS

The present study was conducted on 6 weeks old healthy rats of either sex. The rats were procured from a registered laboratory animal breeder and housed in poly propylene rat animal cages and maintained on balanced laboratory animal feed and clean water *ad libitum* at Animal house, College of Veterinary Science & Animal Husbandry, (Indira Gandhi Krishi Vishwavidyalaya), Anjora, Durg. Rats were acclamatised to the animal house for seven days and were dewormed with Praziplus[1] Tab. @ 5-7.5mg / kg b.wt. orally, given mineral mixture and a course of antibiotic before the start of experiment.

3.3 EXPERIMENT NO. 1 - ACUTE TOXICITY STUDY

Acute toxicity is the toxicity produced by any chemical when it is administered in one or more doses during a period not exceeding 24 hours (CDER, 1996). Acute toxicity study of sodium benzoate in female albino rats was determined by Approximate Lethal Dose (ALD) method as described by Hayes (2001). For this purpose five healthy female rats aged six weeks having weight between 60-100g were taken. All the rats were fasted overnight before dosing. Initially different arbitrary doses (700, 1050, 1575, 2363, 3544 and 5316 mg/kg body weight of sodium benzoate in distilled water) were administered until the lowest lethal dose was obtained. The rats were kept under constant observation for acute manifestation of sodium benzoate toxicity. The administration of the sodium benzoate was carried out orally by using a specially designed gastric needle attached to a syringe in volume not exceeding one ml per rat.

3.3.1 Observation of symptoms and behaviour

Immediately after administration of the sodium benzoate, rats were placed in the cages and they were carefully observed for the nature and severity of the clinical symptoms. Mortality was recorded up to 24 h post administration.

3.3.2.1 Gross pathology

All the rats which died during the experiment were autopsied and were examined for the appearance of lesions if any. Samples of tissues from different organs were collected and fixed in 10 per cent formal saline for histopathological examination.

3.3.2.2 Histopathology

Formal saline fixed tissues from different organs after washing in running tap water were dehydrated in sequences of acetone, cleared in sequences of xyline and embedded in paraffin wax. Paraffin blocks were prepared, sections were cut at 3-5µ and stained with hematoxylin and eosin as per the standard method (Singh and Sulochana, 1997).

3.4 EXPERIMENT NO. 2- SUBACUTE TOXICITY STUDY

3.4.1 Experimental design

A preliminary study was done to determine the dose of sodium benzoate in albino rats for subacute toxicity study. Adult albino rats were divided at random into ten groups of two animals each. Prior to administration of the test material, the animals were weighed individually and were fasted over night. One rat from each group received a single oral dose (25, 50, 100, 200, 400, 600, 800 and 1000 mg/kg body weight of sodium benzoate in distilled water) for 28 days. One rat was kept as control for each of the doses and administered only distilled water.

The dose of 400mg/kg body weight had produced symptoms of toxicity but no mortality and hence was selected as high dose i.e. toxic dose. The dose of 25 mg/kg body weight had produced no observable toxicity and was selected as low dose that is non-toxic dose and the medium dose was chosen as 100 mg/kg body weight.

The rats were acclimatized for a period of one week before the start of oral dosing with sodium benzoate. For this seventy two rats consisting of equal number of male and female rats were divided at random into four different groups (Gr I, Gr II, Gr III and Gr IV) containing 18 rats each (9 Males + 9 Females). Approximate Lethal Dose of sodium benzoate (3544 mg/kg) was taken into consideration for calculation of different dose groups. Rats of Gr II, Gr III and Gr IV were administered sodium benzoate, suspended in distil water, at the dose rate of 25 mg/kg, 100 mg/kg and 400 mg/kg respectively. The design of experiment is summarized in **Table 1**.

Table 1. **Design of experiment of subacute sodium benzoate toxicity in albino rats**

Groups	No. of animals	Dose of sodium benzoate	Volume	Days of observa-tion
Gr I	18 (9M + 9F)	Nil (control Group), only distil water	1 ml	28
Gr II	18 (9M + 9F)	A repeated low dose level which produces no observable toxicity (non toxic dose) N.O.A.E.L	1 ml	28
Gr III	18 (9M + 9F)	A repeated mid dose level (between high and low doses).	1 ml	28
Gr IV	18 (9M + 9F)	A repeated high dose that will produce symptoms of toxicity (toxic dose).	1 ml	28

Sodium benzoate in distil water was administered directly in stomach by using gastric probe attached with 1 ml tuberculin syringe. Rat's body weight was recorded before administration of sodium benzoate. The daily oral administration was continued for 28 days. All the rats were kept in well managed rat cages and free accessed to *ad libitum* standard feed and water.

PARAMETERS STUDIED

3.4.2 Clinical signs

All the rats were observed daily for any toxicity symptoms during entire period of experiment.

3.4.3 Body weight gain

Individual body weights of rats of all the groups were recorded before start of the experiment and thereafter weights were taken at weekly intervals during whole study periods.

3.4.4 Weight of organs

Rats were sacrificed on day 28 and weights of heart, lungs, liver, kidneys, brain, spleen, testes and ovary were taken for calculation of organ weight factor. Organ weight factor was calculated to know the effect of sodium benzoate on growth of various organs of both male and female albino rats. Organ weight factor was calculated by dividing organ weight with body weight (g) and the result was multiplied with 1000.

$$\text{Organ Weight Factor} = \frac{\text{Organ weight}}{\text{Whole body weight}} \times 1000$$

3.4.5 Haematological studies

At the end of the experiment on day 28 blood samples were collected before sacrificing rats for the estimation of haematological parameters. Blood samples were drawn from retro-orbital plexus with the help of capillary tubes as described by Talwar (1983). Heparin (sodium salt) was used as an anticoagulant (10 IU/ml).

Haemogram:

Hematological studies were carried out on the day of collection of blood. Hemoglobin (Hb) content of blood was estimated by Sahli's hemoglobinometer (Coles, 1986). Packed Cell Volume (PCV) was estimated by microhematocrit method (Coles, 1986). Total erythrocyte count (TEC) and Total leukocyte count (TLC) were estimated as per the method described by Jain (1986) by using Gower's solution (s d fine- chem. limited, Mumbai - 400030) and W.B.C. diluting fluid (Merck Limited, Mumbai-400018) respectively. Differential leukocyte count was determined as per the method described by Jain (1986) with slight modification.

3.4.6 Biochemical studies

On day 28 blood samples were collected and plasma was separated for the estimation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine, total protein, albumin, globulin, albumin:globulin ratio, glucose, blood urea nitrogen (BUN) and Cholesterol.

3.4.6.1 Alanine aminotransferase

Alanine aminotransferase or Plasma Glutamic Pyruvate Transaminase level in the plasma was measured by using kit (Siemens Medical Solutions

Diagnostics Ltd., Gujarat) on Chemistry semiauto-analyser RA-50 (Bayer Mac, Germany) as described in literature provided by manufacturer. PGPT level was expressed as U/L.

3.4.6.2 Aspartate aminotransferase

Aspartate aminotransferase or Plasma Glutamic Oxaloacetic Transaminase level in the plasma was measured by using Kit (Transasia Bio-Medicals Ltd.) on Chemistry semiauto-analyser RA-50 (Bayer Mac, Germany) by UV kinetic (IFCC) method as described by manufacturer of the kit. AST level was expressed as U/L.

3.4.6.3 Alkaline Phosphatase

Alkaline Phosphatase level in the plasma was measured by using Kit (Siemens Medical Solutions Diagnostics Ltd., Gujarat) on Chemistry semiauto-analyser RA-50 (Bayer Mac, Germany) by modified PNPP method as described by manufacturer. ALP level was expressed as U/L.

3.4.6.4 Plasma Creatinine

Creatinine level in the plasma was measured by using kit (Siemens Medical Solutions Diagnostics Ltd., Gujarat) on Chemistry semiauto-analyser RA-50 (Bayer Mac, Germany) by Picrate method described in literature provided by manufacturer. Creatinine level was expressed as mg/dl.

3.4.6.5 Plasma Glucose

Glucose level in plasma was estimated by standard glucose kit (Siemens Medical Solutions Diagnostics Ltd.) on Chemistry semiauto-analyser RA-50

(Bayer Mac, Germany) by GOD / POD method as described in literature provided by manufacturer. Glucose level was expressed as mg/dl.

3.4.6.6 Plasma total protein

Total protein level in plasma was estimated by standard kit (Siemens Medical Solutions Diagnostics Ltd., Gujarat.) on Chemistry semiauto-analyser RA-50 (Bayer Mac, Germany) by Biuret method as described in the literature provided by manufacturer. Total protein was expressed as g/dl.

3.4.6.7 Plasma total albumin

Total albumin level in plasma was estimated by standard kit (Siemens Medical Solutions Diagnostics Ltd., Gujarat.) on Chemistry semiauto-analyser RA-50 (Bayer Mac, Germany) by BCG method as described in literature provided by manufacturer. Total albumin was expressed as g/dl.

3.4.6.8 Plasma total globulin

The total plasma globulin was estimated by subtracting total albumin (g/dl) from total plasma protein (g/dl). It was expressed as g/dl of plasma.

3.4.6.9 Albumin globulin ratio

Albumin globulin ratio was measured by dividing the albumin value with the globulin value.

3.4.6.10 Blood Urea Nitrogen

Blood Urea Nitrogen level in plasma was estimated by standard kit (Siemens Medical Solutions Diagnostics Ltd, Gujarat.) on Chemistry semiauto-analyser RA-50 (Bayer Mac, Germany) by UV method as described in the literature provided by manufacturer. It was expressed in mg/dl.

3.4.6.11 Cholesterol

Cholesterol level in plasma was estimated by kit (Siemens Medical Solutions Diagnostics Ltd., Gujarat.) on Chemistry semiauto-analyser RA-50 (Bayer Mac, Germany) by enzymatic method as described in the literature provided by manufacturer. It was expressed as mg%.

3.4.7 Immunological studies

3.4.7.1 Assessment of humoral immune response

Humoral Immune response was assessed by micro Haemagglutination (HA) test as described by Hudson and Hay (1989) with slight modification. The venous blood from the sheep was collected aseptically from the jugular vein of the animal in equal volume of Alsever's solution. It was kept in 2-8^{0}C for a week long period for stabilization. Sheep Red Blood Cells (SRBC) were obtained by centrifugation and washing with phosphate buffer saline thrice at 2000 rpm for 10 minutes. Finally a suspension of 5 per cent SRBC was adjusted with PBS.

For immunization, 1 ml of 5% SRBC was injected intraperitoneally in six rats of each group on day 16 of the experiment. Blood from SRBC injected rats was collected on 28th day without anticoagulant and serum was separated. Serum was kept in water bath at 56^{0}C for 30 minutes to inactivate the complement fraction of the sample. The haemagglutinins produced in response to SRBC was determined by micro HA test. The test was performed in micro titre plates. For this one per cent suspension of SRBC in NSS was used as working suspension. The plate was thoroughly cleaned and each test was carried out in duplicate according to the protocol given in appendix I. The reciprocal of the highest

dilution of serum causing complete haemagglutination was taken as HA titre of the serum sample and expressed in $\log_2$ values.

3.4.7.2 Assessment of cell mediated immune (CMI) response

Dinitroflurobenzene (DNFB) contact skin sensitivity test

Cell mediated immune response; based on delayed type hypersentivity reaction was measured by Dinitroflurobenzene (Sigma Chemical Co. Ltd., U.S.A.) test as described by Phanuphak *et al.* (1974) and later slightly modified by Tamang *et al.* (1988). DNFB test for monitoring cell mediated immunity was done on day 25 after primary sensitization at day 21.

For this test 6 rats were randomly selected from each group. Dorsal aspects of ears of the selected rats were cleaned for the application of DNFB. All the selected rats of each group were sensitized with one drop of 2% DNFB in 4:1 acetone:olive solution to the right ear. The left ear was kept as respective control, on which only the vehicle i.e. the acetone olive solution (4:1) was applied. Four days after primary sensitization, the sensitized rats were challenged with 1% DNFB in 4:1 acetone olive oil solution on day 25. Ear thickness was measured with engineers' micrometer at 0, 6, 12, 24 and 48 hours post challenge.

3.4.8 Pathological studies

3.4.8.1 Postmortem examination and collection of tissue samples

All the rats were sacrificed on 29^{th} day of the experiment. Rats which died during the experiment and the rats which were sacrificed on the last day (29^{th} day) of experiment in the confined disinfected laboratory were subjected to post mortem examination for gross and histopathological alterations.

Post mortem (necropsy) examination was made by systemic approach. Detailed post mortem lesions from all the rats were recorded.

For histopathological examination, the routine procedure adopted at Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Anjora, Durg, were employed. Tissue sections were cut between 3-5 µ and stained with haematoxylin and eosin stain (H&E) method as described by Luna (1968).

3.4.9 Statistical analysis

Statistical analysis was carried out by using complete randomized design (CRD)-single factor analysis of variance by Snedecor and Cochran (1968). The mean values between treatment and control group were tested for critical difference (CD), if any.

3.4.10 Photography

Photography was carried out by using cyber shot DSC- P200 (Sony corp, Japan).

Chapter-IV

Results and Discussion

The various observations made and results obtained of the studies on the effect of short term exposure of sodium benzoate to albino rats in different doses are discussed in this Chapter.

4.1 EXPERIMENT NO. I – ACUTE TOXICITY STUDY

4.1.1 Determination of approximate lethal dose

`The Approximate Lethal Dose of sodium benzoate was determined as 3544 mg/kg body weight in albino rats. Similarly, Duel *et al.* (1954) and Loeser (1977) repoted LD_{50} value of sodium benzoate in rats as 3140 mg/kg body weight. LD_{50} of sodium benzoate has also been reported to be 4070 mg/kg body weight in rats (Smyth and Carpenter, 1948).

4.1.2 Toxic signs and symptoms

Acute toxicity study of sodium benzoate in female albino rats was determined by Approximate Lethal Dose method as described by Hayes (2001). Sodium benzoate was orally administered in graded doses to albino rats (700, 1050, 1575, 2363, 3544 and 5316 mg/kg body weight). The rats given sodium benzoate @ 700 mg/kg body weight showed hyperactivity after 30-45 minutes and lasted for 2 - 4 h of intoxication. The rats, which received 1050 mg/kg body weight of sodium benzoate, exhibited mild depression and hypoactivity. The rats, which were, administered sodium benzoate orally @ 1575 mg/Kg body weight,

showed decreased motor activity, hyperactivity followed with depression and anorexia. The rats, which received 2363 mg/kg body weight of sodium benzoate, showed diarrhoea, muscular weakness and hypoactivity. There was no death of rat.

The rats, which were administered sodium benzoate @ 3544 mg/kg body weight showed depression within 2-3 hours after dosing. Sodium benzoate produced toxic symptoms, which appeared in 2-3 hours following its administration. Gross observable symptoms were depression, dyspnoea, severe respiratory distress, gasping, piloerection, exophthalmus with loss of eye reflex **(Fig. 1)** tremors, diarrhoea with pasting of hairs around anus, extended hind legs **(Fig. 2)** and moribund status etc. In some rats, the toxicity manifestations recorded were emaciation, prostration, opisthotonus, porphyrin staining around eyes and nostrils, ataxia, tonic seizures, tremors **(Fig. 3),** incoordinations and convulsions followed by death after 4 h of intoxication.

Similar findings have been reported in both benzoic acid and sodium benzoate toxicity in rats (Smyth and Carpenter, 1948; Deuel *et al*., 1954 and Bayer, 1977). Jerome Charles *et al*. (2009) reported symptoms of salivation, lacrimation, exophthalmus, piloerection, labored breathing, splay legs, porphyria around eyes and diarrhoea in rats fed carbosulfan in diet.

The rats given sodium benzoate @ 5316 mg/kg body weight showed profuse salivation, incoordination, hyperaesthesia, tonic seizures, tremors, severe respiratory distress and convulsions followed by death after 30 minutes of sodium benzoate intoxication.

Therefore, approximate lethal dose of acute sodium benzoate toxicity in rats was estimated as 3544 mg/kg body weight. Approximate lethal dose was again administered to one rat with one control rat for confirmation. Death of treated rat was observed after 4 hours with similar clinical signs as above.

The above finding of acute toxicity study suggests acute effects due to exposure to sodium benzoate (@ 3544 mg/kg body weight) were mainly neuropathy (effect on the nervous system). It may act on ion channels within the nerve cells to disrupt proper function of the cells of both peripheral and central nervous systems. At lower doses, this may take the form of stable, repetitive firing of the neuron, but high doses may result in depolarization of the nerve cells and blockage of conduction (Ray, 1991) which completely upset the proper functioning of the entire nervous system and eventually result in death (Wouter and Van den Bercken, 1978). The results of the ALD determination are shown in the **Table 2**.

Table 2. Acute toxicity manifestation of sodium benzoate in albino rats

S.No	Dose (mg/kg bwt.)	Toxicity signs	Approx. time of death (h)
1	700	Hyperactivity	-
2	1050	Mild depression	-
3	1575	Decreased motor activity	-
4	2363	Diarrhoea, muscular weakness and hypoactivity	-
5	3544	Incoordination, diarrhoea, extended hind legs, extensive tremors, convulsion leading to death.	4 – 5

4.1.3 Pathology of acute sodium benzoate toxicity

4.1.3.1 Gross pathology

The gross changes observed at necropsy of the rats, which succumbed due to acute sodium benzoate toxicity, were congestion and hyperaemia in most of the organs *i. e.* liver, heart, kidneys, spleen and brain. Severe haemorrhages were recorded in lungs **(Fig. 4).** Reports on acute sodium benzoate toxicity in rats are scanty in the available literature. Death in sodium benzoate toxicity might have occurred due to respiratory failure. The results of the present study are in agreements with Malpe *et al*. (1996) in acute toxicity study of deltamethrin in rats. They reported congestion in vital organs like lungs, liver, spleen, kidney, brain and hearts. Kumar *et al.* (2002) also observed congestion in heart, liver, intestine and stomach in acute toxicity studies of custard apple seed oil in mice. They also reported that kidneys were moderately congested and moderate amount of catarrhal exudate was present in the intestine. Vasodilatation was prominent especially in the subcutaneous and enteric vessels along with patches of pale areas on spleen.

4.1.3.2 Histopathology

Liver

On histopathological examination liver of rats given 3544 mg/kg body weight showed fatty changes **(Fig. 5)** and necrosis of hepatocytes showing pyknotic nucleus **(Fig. 6).** Granular degeneration and vacuolization in hepatic cells have been noticed in rats due to deltamethrin toxicity (Malpe *et al.*, 1996). Focal necrosis and vacuolar degeneration in the periportal hepatocyte were observed in malathion intoxication (Piramanayagam and Monohar, 2002). Fatty

changes and necrosis were also reported by Kumar *et al.* (2002) in acute toxicity studies of custard apple seed oil in mice. Fatty changes, necrosis, fibrosis and atrophy of hepatocytes were also observed in methotrexate toxicity (Free-man Narod, *et al.,* 2006).

Lungs

Microscopic picture revealed severe congestion, oedema and hemorrhages around the pulmonary vessels and interstitial spaces in lungs of rats of acute toxicity study. Also, there were thickening of alveolar septa leading to interstitial pneumonia **(Fig. 7).**

Malpe *et al.* (1996) diagnosed severe congestion and hemorrhages microscopically in lung of rats in deltamethrin toxicity. However, no cellular alteration was detectable by means of optical microscopy in lungs by Pham *et al*. (1984) in acute deltamethrin toxicity in mice, rats and anaesthetized dogs. Bhagat *et al*. (2009) reported congestion and oedematous lung microscopically in broilers in chlorpyriphos toxicity.

Heart

No observable microscopical alterations were observed in heart of rats of acute toxicity study except mild degenerative changes in cardiac muscles **(Fig. 8).** Severe congestion and hemorrhages have been observed in acute deltamethrin toxicity in rats (Malpe *et al.,* 1996). There was mild congestion in the myocardium of the goats (Tamang *et al*., 1991). Microscopically, haemorrhages and oedema were observed in the cardiac muscles of rat given lambda cyhalothrin at 80 mg/kg body weight dose level (Mate, 2007). Increased venous hydrostatic pressure in acute toxicity may be responsible for cardiac oedema (Mitchell, 2006).

Brain

On histopathological examination brain showed mild degenerative and necrotic changes **(Fig. 9).** Mild congestion was found in brain of rats in acute toxicity. According to Richerdson (1981) lipid soluble compounds might dissolve in lipid compartment of neuronal membrane and thereby alter their morphology and function. Congestion and haemorrhages in the meninges and cerebellum were observed in single oral dose toxicity study of α-cypermethrin in rats (Manna *et al.*, 2004). Brain of broiler chicken showed mild congestion and vacuolation intoxicated with chlorpyriphos (Bhagat *et al*., 2009).

Kidneys

Microscopically, kidneys of rats in acute toxicity study revealed wide spread degenerative and necrotic changes in proximal convulated tubules and distal convulated tubules. There were also severe haemorrhages **(Fig. 10)** in intertubular spaces. There were protein casts in the lumen of kidney tubules **(Fig. 11)**. Thapa *et al.* (2009) observed congestion, focal tubular epithelial cell degeneration, interstitial haemorrhages and mono nuclear cell infilteration in kidneys of layer birds intoxicated with aflatoxicosis. Piramanayagam and Monohar (2002) documented atrophy of glomeruli, mononuclear cell infiltration in the interstitium, vacuolar changes in the glomerular tuft and degeneration of tubular epithelium and epithelial cast of rats. Subcapsular haemorrhages and coagulative necrosis of tubular epithelium were noted in kidneys of chicken induced with single dose of imidacloprid toxicity (Kammon, *et al.*, 2009). Garg *et al.* (1992) observed tubular degeneration and proliferative changes in glomeruli as well as in the interstitium in their studies on fluvalinate and fenvalarate toxicosis

in mice. Congestion and necrosis of tubular epithelial cells were observed in acute cypermethrin toxicity in goats (Tamang *et al.,* 1991). Almost similar findings have also been reported in acute fenvalarate toxicity in goats (Mohamed and Adam, 1990), in buffalo calves (Tapase *et al.,* 1994) and in cross bred male calves intoxicated with cypermethrin (Patel, 1996). Haemorrhages above Bowman's capsule and inter lobular space were noted by Kumar *et al.* (2002) in toxicity studies on custard apple seed oil in mice.

The study revealed that sodium benzoate adversely affected the kidneys of rats and could have nephrotoxic effect. The toxic irritant brought to the kidney via blood circulation exerts direct toxic effect on tubular epithelium and may cause anoxia as a result of congestion and reduction in blood circulation (Tamang, 1987). Thus it is possible that sodium benzoate might have direct toxic effect on kidney and or result of anoxia due to congestion.

Spleen

There was severe depletion of lymphocytes in the Malpighian corpuscles of the spleen in acute toxicity of sodium benzoate (@ 3544 mg/kg body weight) in albino rat **(Fig. 12)**.

Marked depletion of lymphoid cells and subsequent necrosis of germinal centers were also seen in rats (Ragothaman, 1991) and in crossbred male calves (Patel, 1996) due to cypermethrin toxicosis. Hypertrophy of the Malpighian corpuscles and hyperplasia of the RE cells were noticed in mice intoxicated with fenvalarate (Tapase *et al.,* 1994). Depletion of lymphocytes was prominent feature in spleen in fenpropathrin toxicity in rats (Bhelonde *et al.,* 2004). Spleen showed

discoloration with congestion of red pulp and haemosiderosis in rats induced with lead toxicity (Satish *et al*., 2009).

Ovary

No noticeable changes were observed microscopically in ovary of the rat in acute toxicity. However, Priya *et al.* (2008) reported sub-active ovaries in rats treated with sodium benzoate @ 25 and 50 mg/kg body weight. Also, inactive ovaries with atretic follicles were observed in rats induced with carbosulfan toxicity (Jerome Charles *et al.,* 2009).

4.2 EXPERIMENT NO.2 – SUBACUTE TOXICITY STUDY

The doses employed in the preliminary study for subacute sodium benzoate toxicity, showed no clinical signs of toxicity upto 50 mg/ kg body weight for 28 days. In the doses of 100 and 200 mg/ kg body weight, severe clinical signs of diarrhoea, anorexia and hypoactivity were observed in sodium benzoate intoxication. The maximum tolerable dose for 28 days in albino rats was 400 mg/ kg body weight. Doses of 600, 800 and 1000 mg/ kg body weight caused mortality in albino rats on day 27^{th}, 25^{th} and 21^{st} respectively after oral exposure of sodium benzoate. Effects of repeated dose sodium benzoate toxicity in albino rats on clinico-haematological parameters, biochemical parameters, humoral and cell mediated immunity and pathological changes are as follows.

4.2.1 Clinical signs

The rats administered sodium benzoate @ 25 mg/kg body weight (Group II) did not reveal any significant visible toxic signs or symptoms, except that they were dull and inactive **(Fig. 13)**, especially after third week of treatment. The rats of control group (group I) remained apparently healthy during the experimental

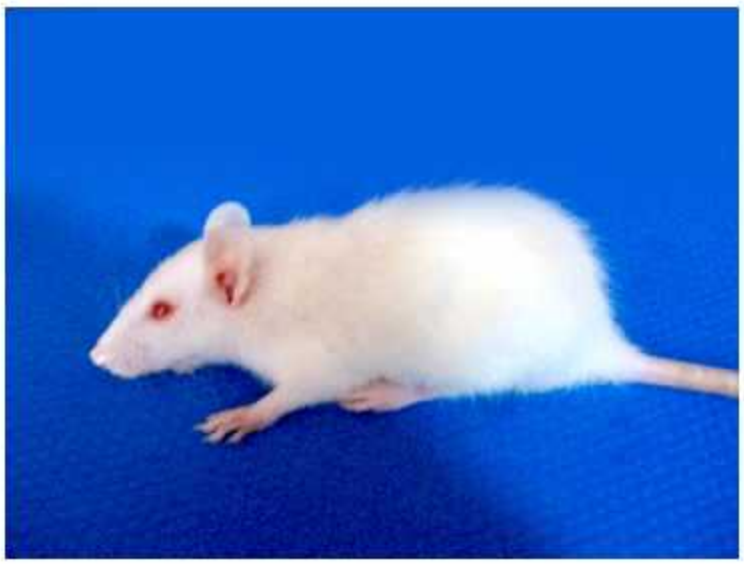


Fig. 1

Fig. 2

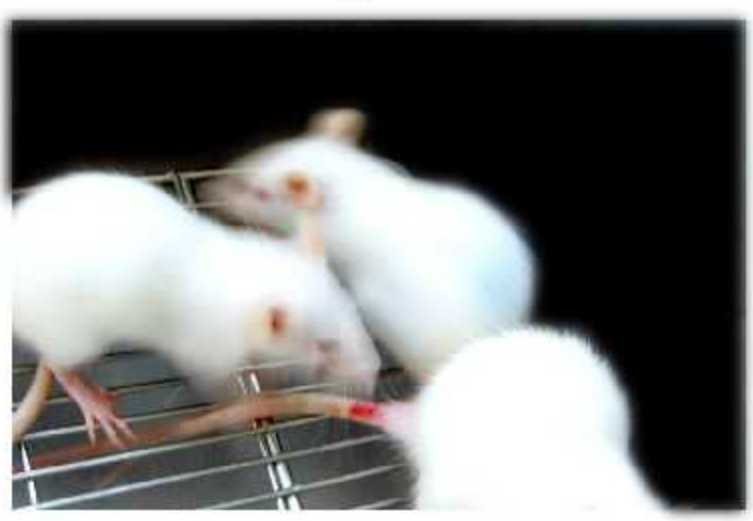


Fig. 3

Fig. 4

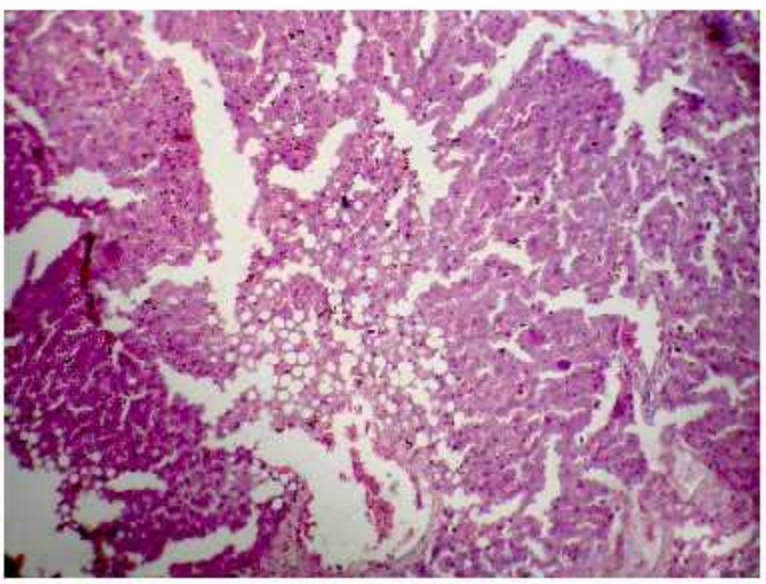


Fig. 5

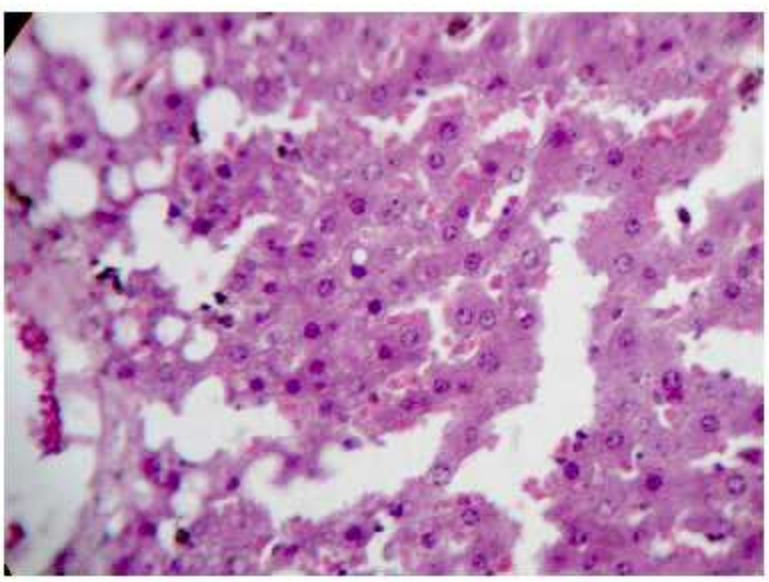


Fig. 6

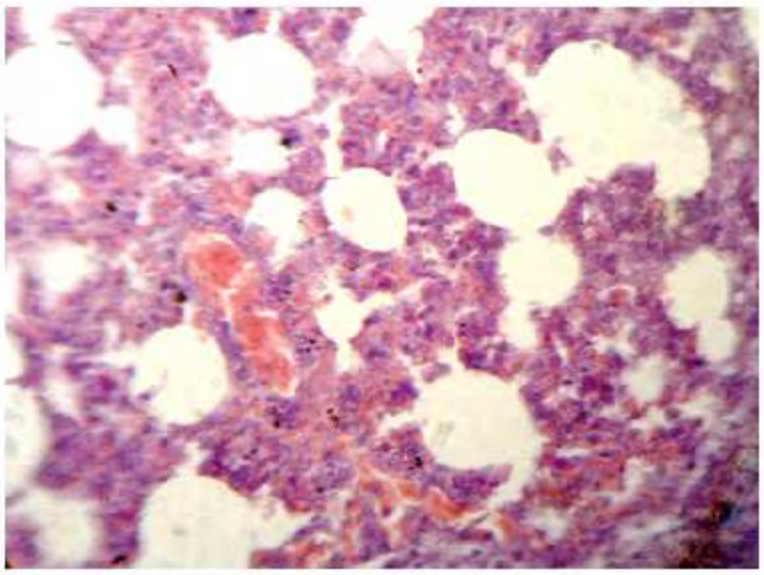


Fig. 7

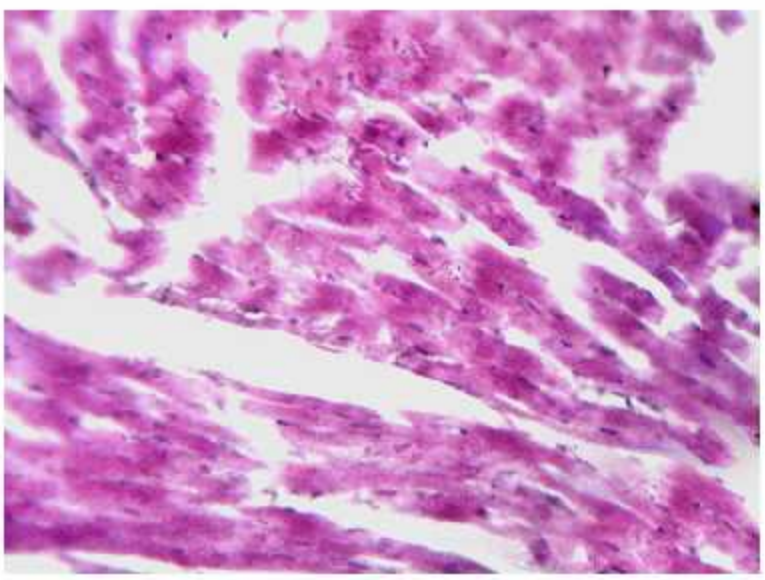


Fig. 8

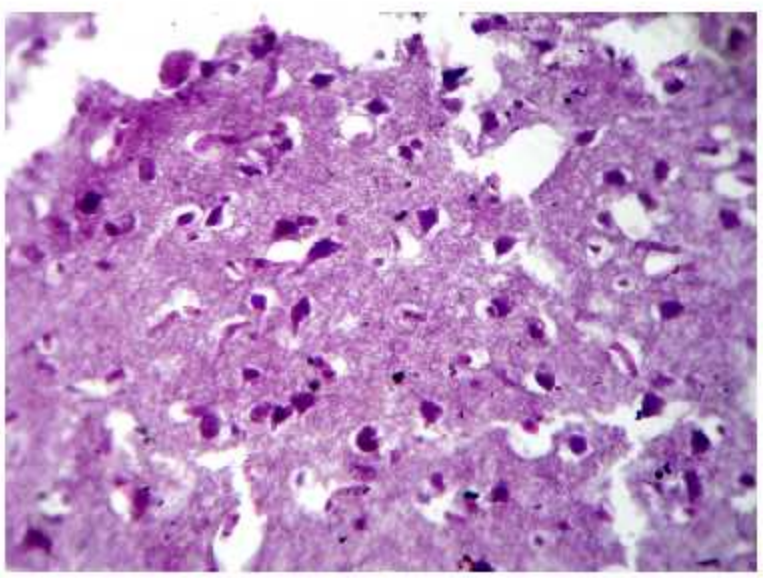


Fig. 9

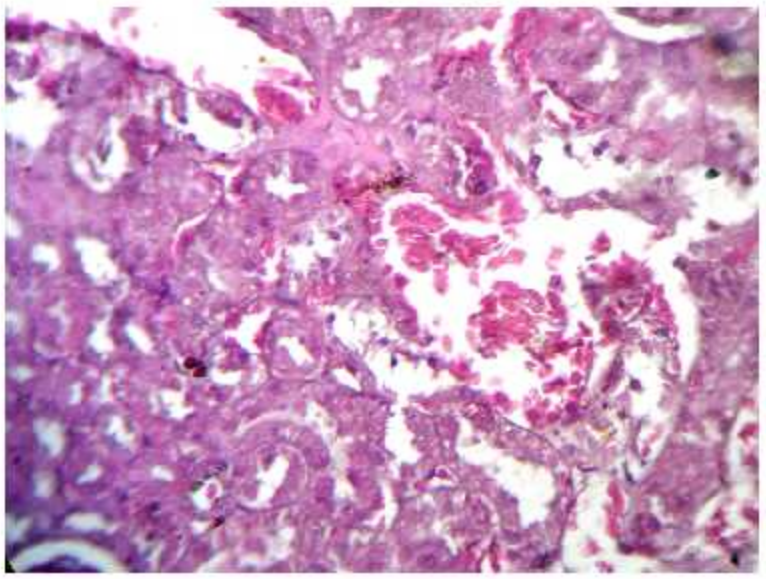


Fig. 10

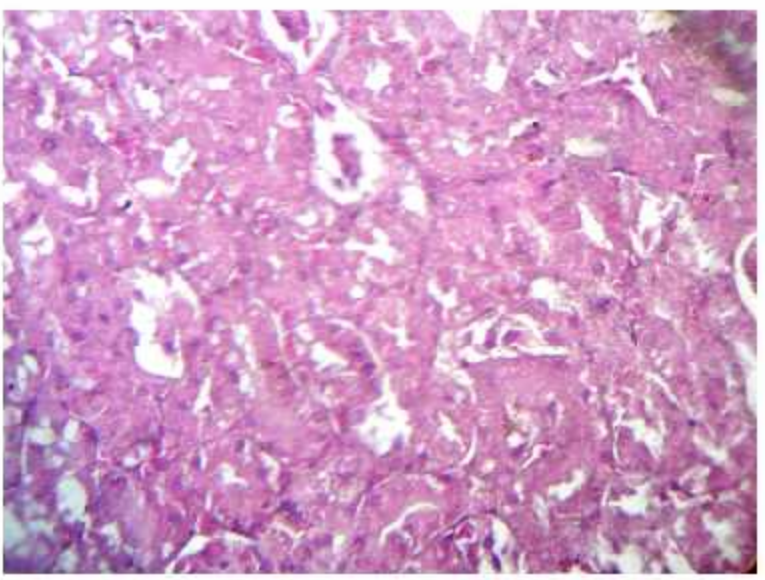


Fig. 11

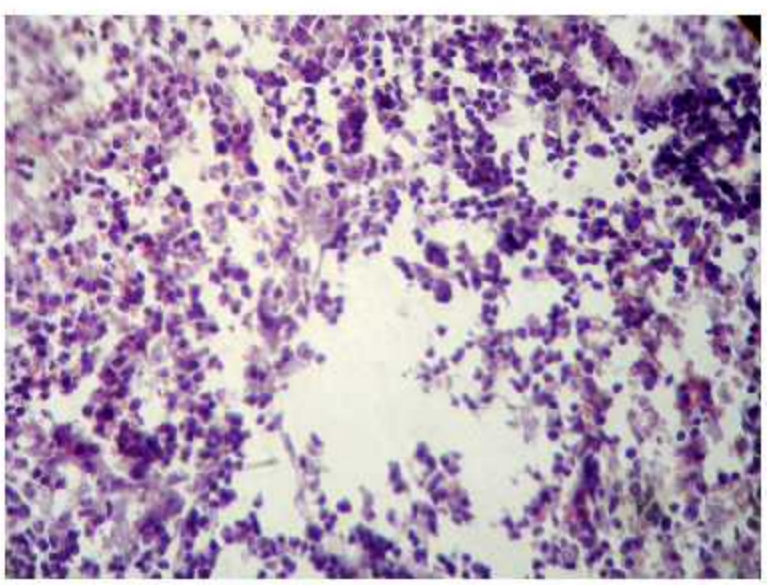


Fig. 12

period. Signs of diarrhoea, anorexia and hypoactivity were observed in the rats of group III (sodium benzoate @100 mg/Kg body weight). Diarrhoea, muscular weakness, hypoactivity, emaciation, exopthalmia, hunched posture **(Fig. 14)** and respiratory distress were observed in the rats of group IV (sodium benzoate @ 400 mg/Kg body weight). The yellow discoloration of urine was also found in the rats of group IV during fourth week of experiment.

Similar observations were seen by Hornychova *et al*. (1995) in supermethrin toxicity in rats. Saxena and Sharma (2000) revealed similar observations with minor variation in rats in subchronic toxicity of fenvalerate in rats. However, Tapase *et al*. (1994) produced fenvalerate toxicity in mice and noticed a transient phase of neurological disturbances followed by a persistent anorexia and intermittent diarrhoea.

4.2.2 Body weight

The average body weight gain observed in various groups at different intervals has been presented in **Table 3** and **Fig. 15**. Feed intake was significantly reduced in the rats of group II, III and IV from day 7 onwards.

From the body weight data it is evident that there is a progressive increase in the weight of albino rats in all the four groups (group I, II, III and IV) during 28 days experimental period. However, dose dependent significant ($P \leq 0.01$) decreases in body weight gain were found in all the treatment groups on day 28 of the experiment. These findings are in accordance with the earlier observations reported by Sodemoto and Enomoto (1980), who recorded a significant reduction of the body weight gain in the rats fed 8% and 4% sodium benzoate in the diet. Moreno (1989) observed reduction in body weight gain in rats fed with sodium

benzoate @ 8% in the diet for 90 days. Similarly, significant changes were found in body weight in F344 rats (0.2, 2.5 and 3.0% of sodium benzoate) and B6C3F1 mice (1.81, 2.09 and 2.4% of sodium benzoate) for ten days by Fujitani (1993). Effects of Benzoic Acid and Sodium Benzoate in chronic exposure animal studies were limited to reduced feed intake and reduced growth in rats and mice (Nair, 2001). Kaboglu and Aktac (2002) also recorded a significant decrease in body weight at 3.0 and 4.0% of sodium benzoate. However, Kieckebusch and Lang (1960), Kowalewski (1960) and Fanelli and Halliday (1963), reported body weight reduction in rats fed with sodium benzoate.

Failure to gain weight could be attributed to loss of appetite and decreased feed consumption due to unpalatability of the feed as it imparts a taste that make the foods unacceptable (Stoewsand, 2000). Loss of vital nutrients and body fluids as a result of watery diarrhoea might have led to stunted growth (Kumar *et al*., 2001). Decrease in body weight gain of rats may also be due to the effect of sodium benzoate on gastro intestinal tract resulting in decreased appetite and absorption. Almost similar findings have been reported in toxicity study of d.d-T80-prallethrin (Seki *et al*., 1987), fenpropathrin (Bhelonde and Ghosh, 2004) and lambda cyhalothrin (Krishnappa *et al*., 2000a) in rats. However, Catinot *et al*. (1989) and Lazarini *et al*. (2001) did not observe any significant changes in the body weight gain of rats exposed to deltamethrin toxicity.

4.2.3 WEIGHTS OF ORGANS

The effects of subacute exposure of sodium benzoate in different doses on liver, kidney, brain, heart, spleen, lungs, ovary, testes, prostrate and adrenal glands are presented in **Table 4**.

Table 3. Effect of daily oral administration of sodium benzoate for 28 days on body wt. (g) in albino rats [n = 18]

Groups	Body Weight				
	0 day	7 day	14 day	21 day	28 day
Gr I	65.78± 1.34 [a]	78.67± 3.31 [a]	84.33± 1.62 [a]	95.78± 2.19 [a]	110.94± 3.30 [a]
Gr II	63.56± 3.21 [a]	73.33± 4.13 [b]**	78.08± 3.17 [ab]	81.11± 3.51 [b]**	84.89± 2.58 [b]**
Gr III	65.22± 3.39 [a]	71.33± 3.32 [ab]*	74.0± 3.05 [bc]**	77.11± 4.14 [bc]**	79.78± 4.34 [b]**
Gr IV	63.33± 1.66 [a]	67.44± 1.83 [b]**	68.44± 2.24 [c]**	70.06± 2.56 [c]**	75.44± 2.72 [b]**

Superscripts may read column wise for comparison of means. Similar superscripts showing means do not differ Significantly. (*P≤0.05), (**P≤0.01)

There were significant (P≤0.05) increase in the weights of liver **(Fig. 16)** of the rats of group III and IV compared to the liver of rats of control group (group I). The present findings are in agreement with the findings of Fujitani (1993), who reported significant increase in the relative liver and kidney weight in male rats received sodium benzoate @ 2.4% in the diet for 10 days and a significant increase in the absolute liver weight in male mice received sodium benzoate @ 3% in the diet for 10 days.

In previous studies, increasing of total liver weight were seen with oral administration of sodium benzoate (Kaboglu and Aktac, 2002) and citric acid (Aktac *et al*., 2002) but it was not significant. Similarly, the effects of BHT on increasing of the liver weight in rats were also shown by McFarlene *et al*. (1997). Seki *et al.* (1987) reported significant increase in weights of liver and kidney in d.d-prallethrin toxicity in rats.

The close relation between sodium benzoate and liver is due to the fact that more than 80% of ingested sodium benzoate is metabolized in the liver. The relation between hepatic tissue damage and elevation of liver enzymes is well documented (Sidhu *et al*., 2004). Increase in the cell size could be the basis of ballooning of hepatocytes (Sheeja *et al*., 2006).

Significant (P≤0.05) increase was found in the weight of kidneys **(Fig. 17)** of rats belonging to group III and IV as compared to rats of group II and control group. These findings are in accordance with Fujitani (1993). Increased relative weight of kidneys might have occurred due to haemorrhage and congestion in sodium benzoate treated rats.

Fig. 13

Fig. 14

Weights of brain were increased significantly (P≤0.01) in rats of group III only. No significant changes were observed in the weight of heart, lungs, spleen, ovaries and testes of rats of any of the treatment groups. Kunimatsu *et al*. (2002) observed no change in weight of testes in esfenvalerate, fenvalerate and permethrin toxicity. In contrast to our studies Mani *et al*. (2002) found significant reduction in the weight of testes in fenpropathrin toxicity in rats.

4.2.4 HAEMATOLOGICAL STUDIES

The results of the effect of different concentrations of orally fed sodium benzoate for 28 days on hematological parameters (TEC (10^6/cu.mm), Hb (g/ dl), PCV (%), MCV (fl), MCH (pg), MCHC (g/ dl), TLC (10^3/cu.mm) and DLC(%)) are shown in **Table 5**.

The oral administration of sodium benzoate for 28 days caused significant (P≤0.01) reduction in the levels of TEC **(Fig. 18)** and TLC **(Fig. 24)** of rats of treatment groups (II, III and IV) compared to rats of control group (group I). PCV measures the percentage by volume of packed RBC in a whole blood sample after centrifugation (Wynne and Edwards, 2003). The study revealed non-significant changes in PCV **(Fig. 19)** of rats of treatment group (II, III and IV) compared to rats of control group (group I).

Effect of hemoglobin on all the four groups has been depicted in **Fig. 20**. Gradual and significant (P≤0.01) decline in hemoglobin concentration was observed in rats of group III and IV compared to rats of group I and group II. Also there was a significant (P≤0.05) reduction of hemoglobin concentration in rats of group II as compared to control group.

The percent values of DLC of rats of all the groups have been shown in **Table.5**. There was dose dependent non-significant increase in neutrophil **(Fig. 25)** percentage and non significant gradual decrease in lymphocyte **(Fig. 26)** percentages in rats of treated group as compared to the rats of control group. No significant alterations in the mean values of eosinophil, monocyte and basophil count in rats of all the groups were observed.

There were no significant differences in MCHC **(Fig. 21)** and MCH **(Fig. 23)** values on rats administered sodium benzoate orally compared to the rats of control group. The mean values of MCV **(Fig. 22)** were found to be significantly ($P \leq 0.01$) higher in rats of group III and group IV as compared to rats of control group.

A significant correlation with diagnostic value has been demonstrated between RBC, Hb, PCV and the blood indices (MCV, MCH and MCHC) in both human and rats (Arechor *et al*., 1982 and Bain, 1989). The findings of significant decrease ($P \leq 0.05$) in hemoglobin concentration along with reduced TEC count might be due to sodium benzoate intoxication suggest the induction of anaemic condition in the rats (Schalm *et al*., 2000; Cheeseborough, 1991 and Ibekwe *et al*., 2007a). A significant decrease in the mean values of TEC and Hb with no alterations in PCV in the treated groups indicates that the sodium benzoate could produce macrocytic hypochromic anaemia if administered orally for 28 days.

Diffuse liver disease (toxic) associated with an anemia is attributed to hypofunction of the marrow. Concomitant foliate deficiency and iron deficiency can also contribute to anemia. Most often, erythroid progenitors are preferentially affected, depression of the white cell count and platelets is less common. The

anemia is often slightly macrocytic due to lipid abnormalities associated with liver failure, which cause red cell membrane to acquire phospholipid and cholesterol in the periphery (Aster, 2006).

The observed significant decrease in WBC levels in sodium benzoate treated group portrays possible succeptibility to infection as WBC perform important role in defending against infection (Schalm *et al*., 2000). Leucocytopaenia associated with lymphocytopaenia has also been reported previously in rats (Wangikar, 2002), rabbit (Mir, 1998), guinea pigs (Thacker and Carlton, 1977), turkeys (Chang *et al*., 1981), chicken (Singh *et al*., 1990) and Japanese quail (Farshid and Rajan, 1995). There were non-significant and mild neutrophilia along with lymphopenia observed in sarcoptic mange infested dogs treated with oral Ivermectin (Chhabra and Bhardwaj, 2009). Similar findings was found by Wangikar (2002) and Satheesh *et al.* (2005) who observed, reduction in leucocytic counts marked by lymphocytopaenia with relative increase in the neutrophil percentage in wistar rats fed with ochratoxins @ 4ppm in the diet. The changes in levels of erythrocyte, red blood index and lymphocytes (lymphopenia) can be an indicator of anemia and immune system deficiency, respectively (Banaee, *et al*., 2008). Mild leucocytopaenia observed in this study was essentially lymphocytopaenic type, which could be associated with lymphoid depletion from lymphoid organs as observed histologically (Satheesh, 2003) and reduced cell mediated and humoral immune responses. Significant increase in MCV values in group III and group IV treated rats suggests hemorrhagic anaemia leading to macrocytic RBCs in the circulation (Cynthia and Kahn, 2005b).

However, no changes in haematological parameters were found by Parker *et al*. (1984) in fenvalerate toxicity in rats.

4.2.5 BIOCHEMICAL STUDIES

4.2.5.1 Plasma aspartate amino transferase, Plasma alanine amino transferase and Plasma alkaline phosphatase

Table 6 and **Fig. 27** illustrate the effect of orally fed sodium benzoate on AST levels in blood plasma in rats. There was significant ($P \leq 0.05$) increase in the level of plasma AST of rats of group IV compared to rats of control group. However, in the rats of group II and group III the increase in the level of plasma AST was found to be non-significant compared to rats of control group.

Effects of daily oral administration of sodium benzoate on plasma ALT in rats of experimental groups are presented in **Table 6** and **Fig. 28**. The activity of ALT was increased progressively and significantly ($P \leq 0.05$) in rats of group III and group IV compared to rats of control group.

Results on the sodium benzoate exposure of daily oral administration for 28 days to albino rats on plasma ALP are shown in **Table 6** and **Fig. 29**. The activity of plasma ALP increased significantly ($P \leq 0.05$) in group IV rats compared to rats of control group.

Simillarly, Ibekwe *et al.* (2007) reported significant and dose dependent increase in AST and ALP level in rats fed sodium benzoate @ 30, 60 and 120 mg/Kg body weight orally. Priya *et al*. (2008) also observed increase in AST, ALT, ALP, BUN and creatinine in both male and female rats of 25 and 50 mg sodium benzoate/ kg body weight groups. The serum enzymes such as AST, ALT

and ALP are the biomarkers of liver pathology and the elevated concentrations of these enzymes in blood/ plasma is due to their leakage from liver cytosol in to the blood stream as consequences of damage to the hepatocytes (Denman *et al*., 1983; Tennant, 1997). Raina *et al*. (1991) suggested that elevation in the activity of AST, ALT and ALP could be attributed to circulatory and degenerative changes in liver and kidneys followed by subsequent release in the blood circulation. Activity of AST is high in acute and chronic liver injury (Tennant, 1997). Elevation in the AST, a mitochondrial enzyme, can be associated with cell necrosis involving the skeletal or cardiac muscle and / or the hepatic parenchyma (Kaneko, 1980). Lorh (1975) reported a considerable increase (251 mU/ml) in serum AST level in chicken suffering from fatty liver kidney syndrome. The present study are in agreement with Chandra *et al*. (1984) who also observed increase in AST and ALT level after feeding the chickens with high protein diets. Koutsos *et al.* (2001) also observed increase in AST with increased crude protein in diet. Bailey *et al.* (1989) observed that adding protein to the diet increased the activity of the serum AST, creatine, kinase and ALP.

ALT, a key cytoplasmic enzyme present in liver and other cells is particularly useful in measuring hepatic necrosis, especially in small animals (Cornelius, 1989). Alanine amino transferase is found elevated in non specific, soft tissue damage (Forbes, 2001).

Alkaline phosphatase, a brush border enzyme is known to be involved in a variety of activities such as permeability, growth and cell differentiation (Lobel and Levy, 1968; Shaffi *et al*., 1974). Alkaline phosphatase (a plasma membrane enzyme) occurs in duodenum and kidney but increases in many organs with

Table 6. Effect of daily oral administration of sodium benzoate for 28 days on plasma enzyme in albino rats

Groups	AST (u/l)	ALT (u/l)	ALP (u/l)
Gr I	**45.0± 5.96** [a] **(6)**	**38.17±4.66** [a] **(6)**	**41.83±3.43** [a] **(6)**
Gr II	**50.17± 4.02** [ab] **(6)**	**44.17±2.63** [ab] **(6)**	**83.17±8.86** [ab] **(6)**
Gr III	**55.67±1.86** [ab] **(6)**	**53.67±4.14** [b]* **(6)**	**117.33±22.93** [ab]* **(6)**
Gr IV	**65.17±3.84** [b]* **(6)**	**70.5±3.39** [c]** **(6)**	**130.17±29.46** [b]** **(6)**

Values indicate Mean ± S.E. Figure in bracket indicate 'n'. Superscripts may read column wise for comparison of means. Similar superscripts showing means do not differ significantly. (*P≤0.05), (**P≤0.01)

increase cellular activity and cellular damage. Raised levels, indicate non specific tissue irritation. Pathological elevations are commonest in osteoclastic and liver disease (Sturgill and Lambert, 1997 and Forbes, 2001). Zimmarman and Henery (1969) suggested that any damage to liver, small intestine, bone and kidneys might cause increase in the level of ALP in the blood stream.

Elevation of enzyme activity in sodium benzoate treated rats suggests widespread tissue damage including liver damage as it is the primary organ of biotransformation of sodium benzoate and alterations in membrane structure.

4.2.5.5 Glucose

The plasma glucose level in rats of sodium benzoate treated groups showed non-significant decrease as compared to the rats of control group (**Table 7** and **Fig. 30**).

Similarly, Priya *et al* (2008) reported hypoglycemia, hypercholesterolemia and hypoproteinemia in rats treated with sodium benzoate orally @ 25 and 50 mg/Kg body weight. However, no change in glucose level was reported by Krishnappa *et al*. (2000) in lambda cyhalothrin 2.5% EC in rats.

In the present study hypoglycemia was observed in rats on 28^{th} day and there was a dose dependent decrease in glucose concentration after 28 day of oral administration of sodium benzoate. The result of the present study suggested that thyroid could be sensitive to sodium benzoate.

4.2.5.6 Total Protein, Albumin, Globulin and Albumin globulin ratio

The effect of subacute exposure of sodium benzoate in different doses on plasma total protein, albumin, globulin and albumin globulin ratio of albino rats are presented in **Table. 7**.

There were significant ($P \leq 0.01$) increase in the levels of plasma total protein in rats of both group III and IV on day 28 but there was no significant effect on plasma total protein in rats of group II as compared to rats of control group **(Fig. 31)**.

Significant ($P \leq 0.05$) increase in the plasma albumin level was found in rats of group IV compared to rats of group III **(Fig. 32)**. There were no significant differences on the plasma albumin levels of rats of treated groups compared to rats of control group.

Non-significant decrease on plasma globulin levels **(Fig. 33)** and plasma albumin globulin ratio was observed in the rats of group II, III and IV compared to the rats of control group. There was only non-significant decrease in albumin globulin ratio in rats of group IV compared to the rats of control group.

The present findings are in accordance with the findings of Fujitani (1993), who reported changes in serum levels of cholesterol, albumin and total protein in male rats fed with sodium benzoate. However, Ibekwe *et al.* (2007) found no significant changes in plasma protein concentration of male albino rats administered sodium benzoate @ 30, 60 and 120 mg/Kg body weight orally. Priya *et al*. (2008) observed hypoproteinemia and hypoglobulinemia in sodium benzoate treated rats. A significant decrease in total serum protein, globulin and an increase

Table 7. Effect of daily oral administration of sodium benzoate for 28 days on plasma metabolites in albino rats [n= 6]

Parameters	Group I	Group II	Group III	Group IV
Glucose (g/dl)	63.69±3.15 [a]	57.71±15.95 [a]	50.39±7.17 [a]	40.86±1.10 [a]
Total Protein (g/dl)	7.7±0.36 [a]	8.55±0.27 [ab]	8.93±0.37 [b]**	9.33±0.19 [bc]**
Albumin (g/dl)	4.27±0.22 [ab]	3.67±0.13 [a]	3.62±0.24 [a]	4.55±0.36 [b]
Globulin (g/dl)	3.43±0.43 [a]	3.6±0.66 [a]	4.93±0.38 [a]	2.95±0.58 [a]
Albulin : Globulin	1.37±0.21 [a]	1.25±0.26 [a]	0.76±0.09 [a]	2.66±1.22 [a]
Creatinine (mg/dl)	0.98±0.19 [a]	1.18±0.11 [a]	1.87±0.2 [b]**	2.15±0.19 [b]**
BUN (mg/dl)	20.62±1.33 [a]	21.02±.77 [a]	21.78±0.81 [a]	22.35±0.7 [a]
Cholesterol (mg/dl)	44.15±2.25 [a]	55.72±3.00 [b]**	64.39±2.85 [bc]**	68.72±2.40 [cd]**
Uric acid (mg/dl)	1.8±0.30 [a]	2.06±0.18 [a]	2.25±0.15 [a]	2.42±0.11 [a]

Values indicate Mean ± S.E. Superscripts may read row wise for comparison of means. Similar superscripts showing means do not differ significantly. (*P≤0.05), (**P≤0.01)

in A:G ratio were observed in lindane, monocrotophos, carbofuran and fenvalerate toxicity in lambs (Khurana *et al.,* 1997). However, Tian (1993) reported significantly increased albumin and total protein in 20 workers exposed to fenvalerate insecticide.

As pointed out by Allison (1955), total serum protein or serum albumin is an indication of the protein reserves in an animal. Increase in albumin and total protein may be due to elevation of stress, decrease protein catabolism and decreased inflammation causing movement of fluid along with proteins in to vascular system (Ghuman *et al*., 1996).

Decreased levels of globulin indicate that the immune competence of the animals would be easily compromised. As a matter of fact, lymphopaenia accompanied by low globulin level, may lead to immunosuppression. In case of decreased globulin level, diseases characterized by deficiency of immunoglobulin, such as agammaglobulinaemia selective IgM, IgA and IgG deficiencies, and transient hypogammaglobulinaemia, may lead to low level globulin (Duncan *et al.,* 1994). Sodium benzoate might possess immunosuppressive properties.

4.2.5.7 Creatinine

Plasma creatinine level was significantly ($P \leq 0.01$) increased in the rats of group III and group IV as compared to the rats of control group (**Table 7** and **Fig. 34**).

Blood levels of non protein nitrogenous substances like blood urea nitrogen and creatinine are elevated in blood when renal function is below 30% of its original capacity and their quantitative estimation indicates the status of renal

function (Dudley *et al.,* 1985; Hu *et al.,* 1991; Finco, 1997). Kidneys are major route of sodium benzoate excretion (Bridges *et al.,* 1970). Significant increase in blood creatinine level may be correlated to the nephrotoxicity or skeletal muscle necrosis or atrophy of skeletal muscle (Pennington, 1971).

Our findings are in accordance with El- Demerdash *et al*. (2004), who revealed significant increase in plasma creatinine and BUN concentration in rats treated with fenvalerate. Similar observation was noted by Yousef *et al*. (2003) in male rabbits in cypermethrin toxicity.

Plasma creatinine level increases with decrease in glomerular filtration rate. Therefore, the present observation indicates that feeding sodium benzoate resulted in renal impairement in the rats. Histopathological sections of kidney also support the present finding and reveales necrosis of glomerular tuft, haemorrhages and severe congestion of blood vessels with presence of albuminous cast in some of the PCT and DCT of kidneys.

4.2.5.8 Blood urea nitrogen

The effects of sodium benzoate exposure after daily oral administration for 28 days to albino rats on blood urea nitrogen are shown in **Table 7** and **Fig. 35**. There was non-significant increase in blood urea nitrogen levels in a dose dependent manner in sodium benzoate treated rats as compared to rats of control group.

Increase in blood urea nitrogen indicates that sodium benzoate has toxic effect on glomerular filtration, carbohydrate deficiency due to anorexia, catabolic states and dehydration (Cynthia and Kahn, 2005). Our findings are in accordance

with El- Demerdash *et al*. (2004), who revealed significant increase in plasma creatinine and BUN concentration in rats treated with fenvalerate. Similar observation was noted by Yousef *et al*. (2003) in male rabbits in cypermethrin toxicity.

Highly significant increase in blood creatinine may be correlated to the renal damage or skeletal muscle necrosis or atrophy of skeletal muscle (Pennington, 1971). Azotemia is a biochemical abnormality that refers to an elevation of the BUN and creatinine levels and is related largely to a decreased glomerular filtration rate. Azotemia is produced by many renal disorders (Alpers, 2006).

4.2.5.9 Cholesterol

The mean values of plasma cholesterol level for each experimental group are presented in **Table 7** and **Fig. 36**.

The activity of plasma cholesterol level increased significantly ($P \leq 0.01$) in rats of group III and group IV as compared to the rats of control group. However, non-significant increase in plasma cholesterol level was observed in rats of group II.

Hassan *et al*. (1988) also observed a significant increase in the level of cholesterol in rabbits intoxicated with decamethrin. Similar trend of hypercholesterolemia have also been seen in goats (Mohmed and Adam, 1990), buffalo calves and mice (Tapase, 1994) due to fenvelarate toxicity. Patel *et al*. (1996) also reported significant increase in the serum cholesterol of crossbred male calves intoxicated with cypermethrin. Ayub Shah and Gupta (1997)

observed a marginal increase in serum cholesterol level in rats due to low doses (@ 24-54 mg/kg body weight) of permethrin. However, no significant changes in the levels of cholesterol have been observed in goats in fenvelarate (Mandal *et al*., 1992) and in rats in lambda cyhalothrin (Krishnappa *et al*., 2000) intoxication.

Elevated level of cholesterol may be an indication of risk of artherosclerosis, biliary diseases, hypothyroidism, starvation, high fat diets or protein losing nephropathy (Forbes, 2001 and Cynthia and Kahn, 2005b). Oser (1976) postulated that biliary obstructions, regardless of the cause may result in hypercholesterolaemia. Mild degree of bile duct hyperplasia could be one of the possible reasons for bile duct obstruction leading to hypercholesterolaemia (Patel, 1996). Hassan *et al*. (1988) suggested that the disruption of the formation of lipoprotein is one of the factors leading to accumulation of cholesterol. Hypercholesterolaemia may also occur in lipolysis or mobilization of reserve fats (Patel, 1996). But in the present study proliferation of the bile duct could not be seen as a constant finding. It seems that hypercholesterolaemia in the present study might have occurred due to the reasons as suggested by Hassan *et al*. (1988), hypothyroidism and starvation.

4.2.5.8 Uric acid

The mean values of plasma uric acid level for each experimental group are presented in **Table 7** and **Fig. 37**. There was non-significant increase in plasma uric acid in a dose dependent manner in sodium benzoate treated rats as compared to the rats of control group.

In the present study increased plasma uric acid concentration indicated increased catabolism of protein and impairment in excretion of uric acid as a result of pathomorphological changes in kidney observed histologically.

4.2.6 Immunological study

In recent years considerable interest has arisen in the immunotoxicological evaluation of agrochemicals. In view of this, the study was also undertaken to rule out the possible effect of subacute sodium benzoate intoxication on the immune system of rats. The immunotoxicological investigation on sodium benzoate revealed an immunosuppressive effect; both cell mediated and humoral immune response of rats.

4.2.6.1 Humoral immune response

The mean values (log_2) of HA titers in rats of all four groups recorded have been given in **Table 8** and depicted in **Fig. 38**

Table 8. Effect of sub acute sodium benzoate toxicity on humoral immune response against SRBC in albino rats on day 28 (n=6)

Parameter	Experimental Groups			
	Group I	Group II	Group III	Group IV
HA Titre (log_2 values)	9.33±0.49 [a]	7.17±0.48 [b] **	6.5±0.43 [b] **	4.67±0.71 [c]**

Superscripts are read row wise for comparison of means with different superscripts differ significantly (*P≤0.05) (**P≤ 0.01).

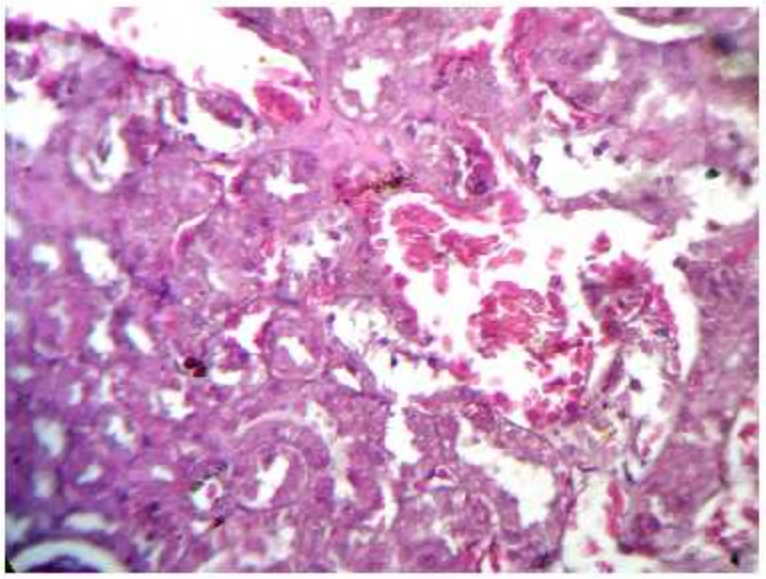
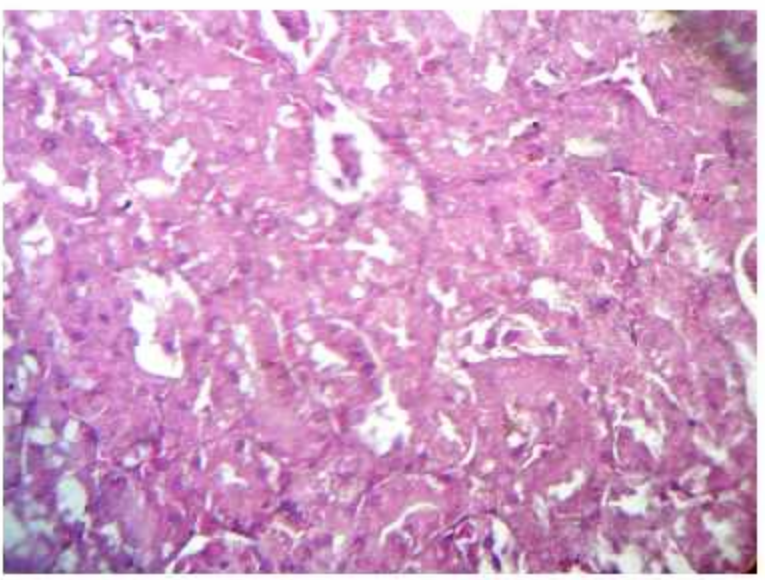


Fig. 10



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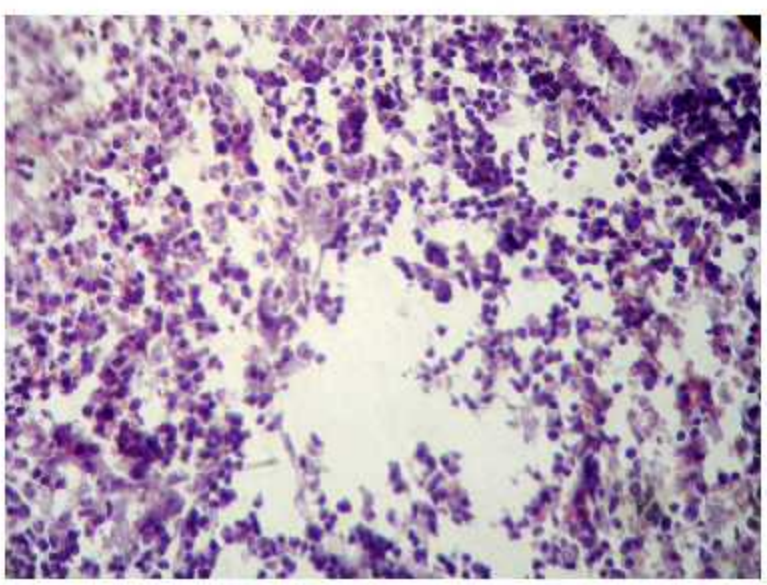
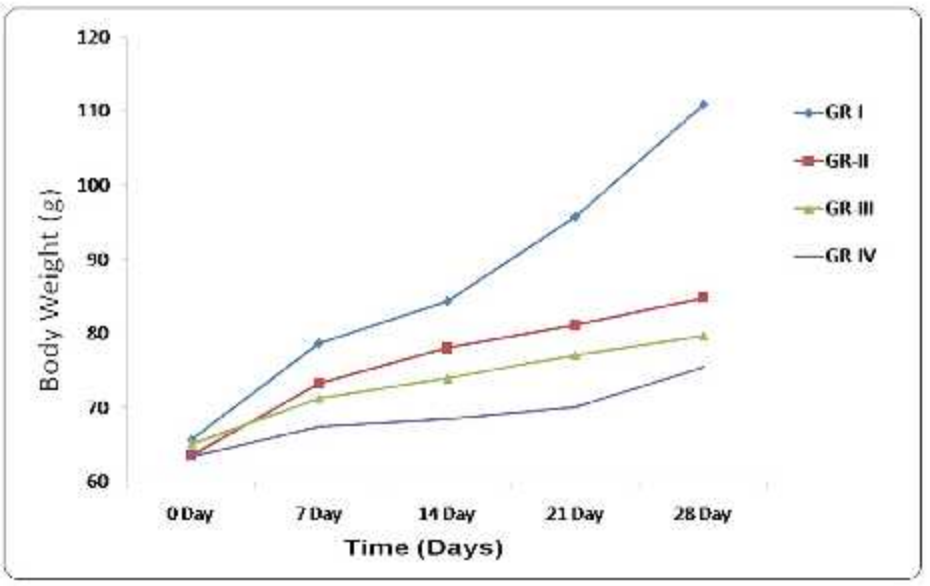


Fig. 12



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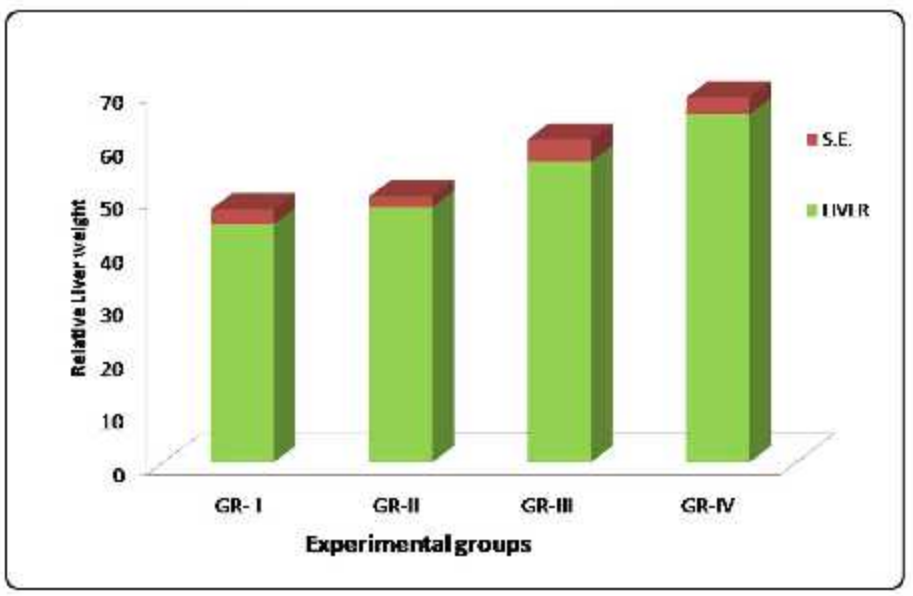

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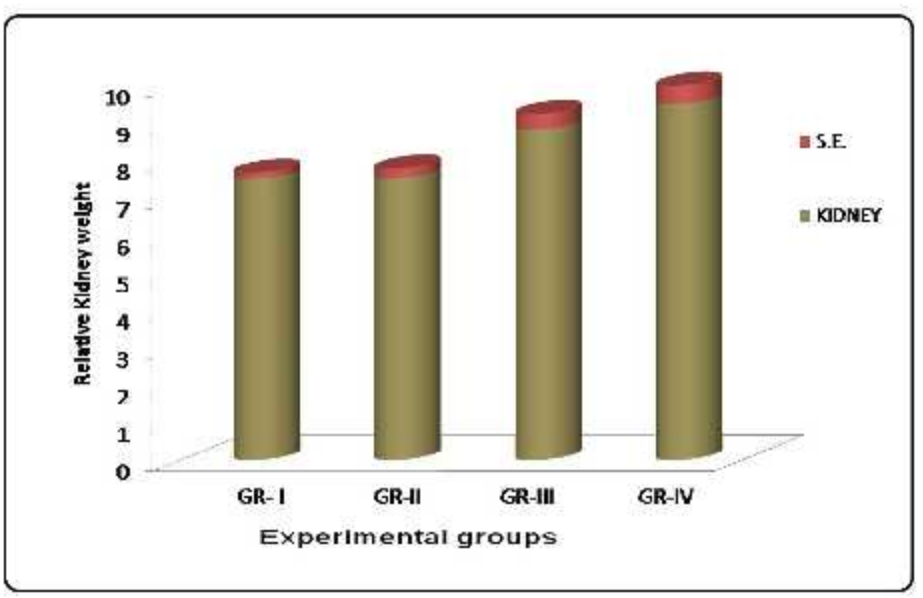

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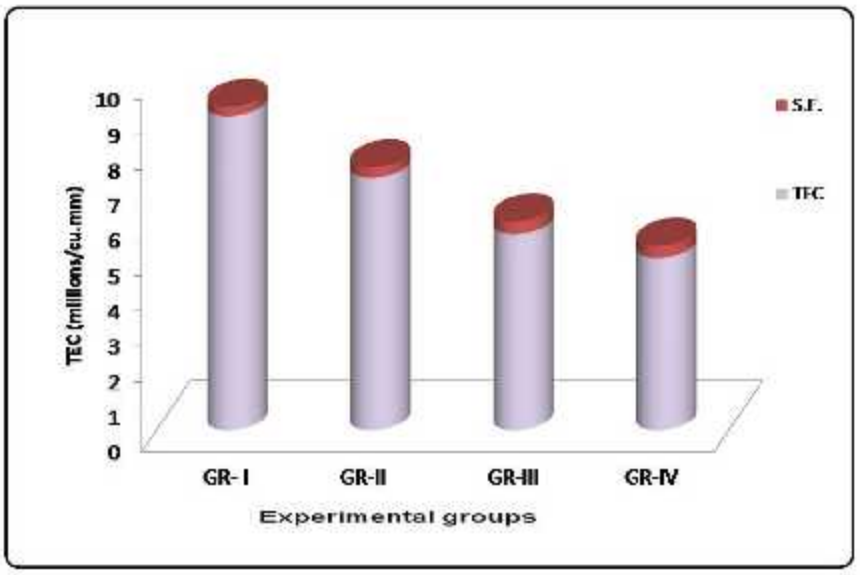

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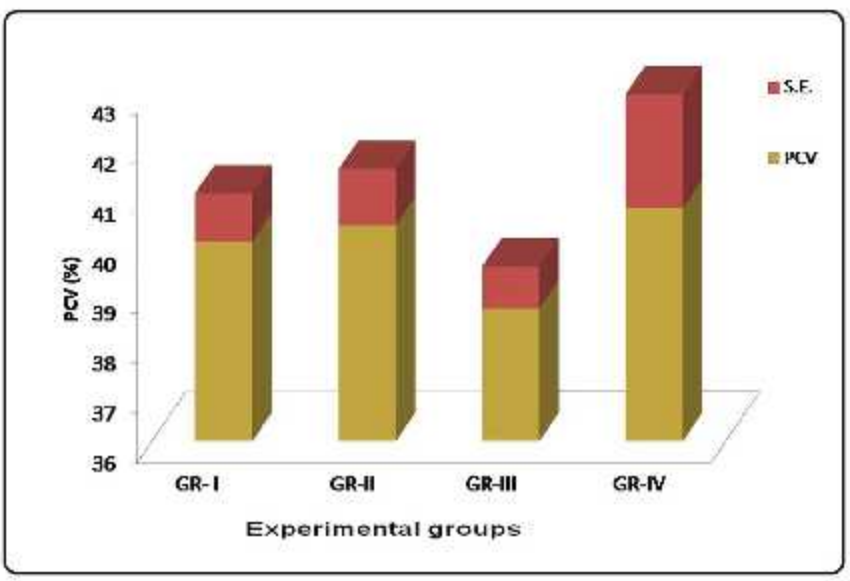

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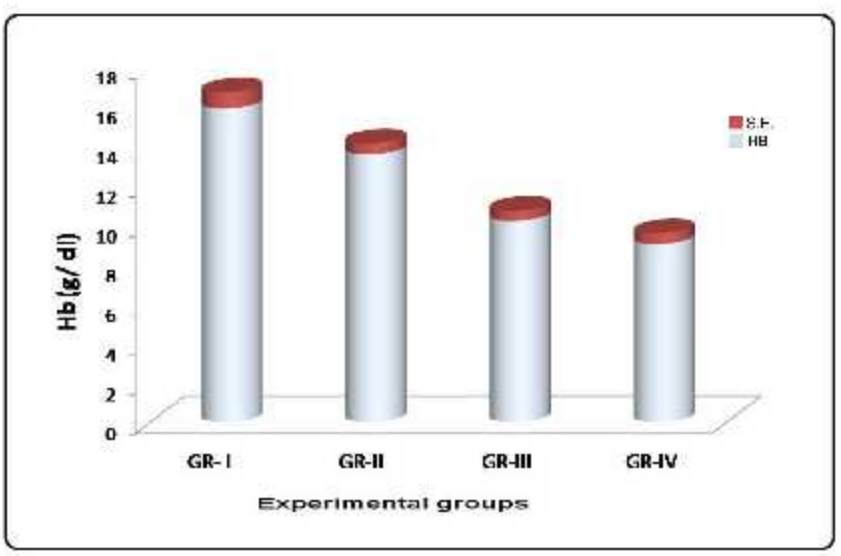


Fig.20

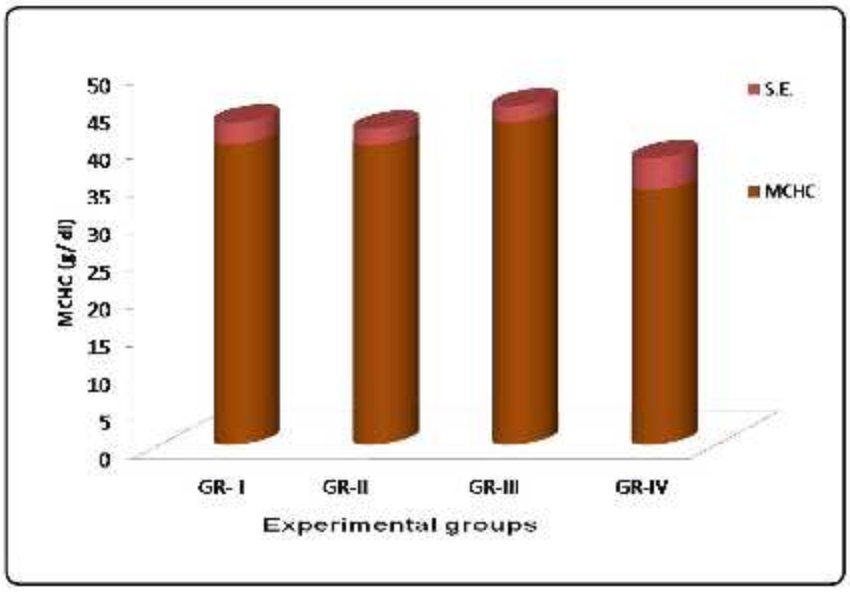


Fig.21

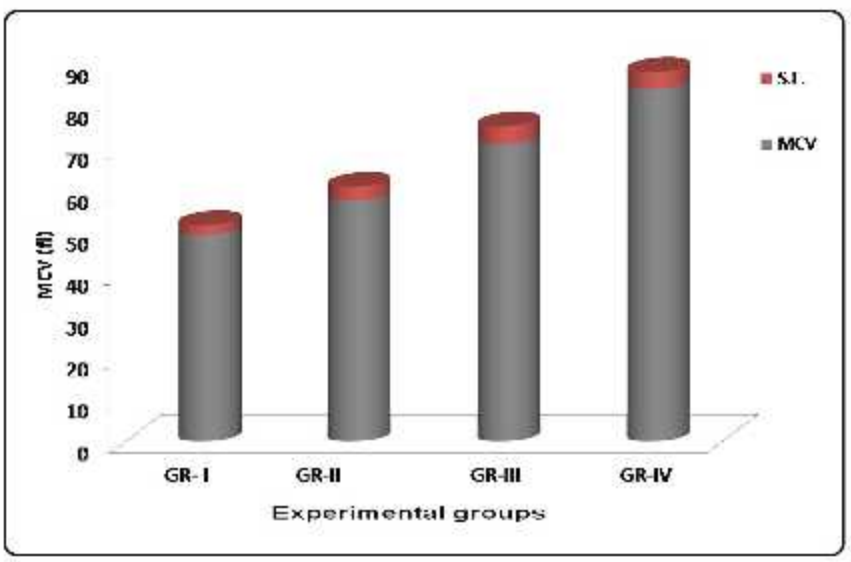


Fig.22

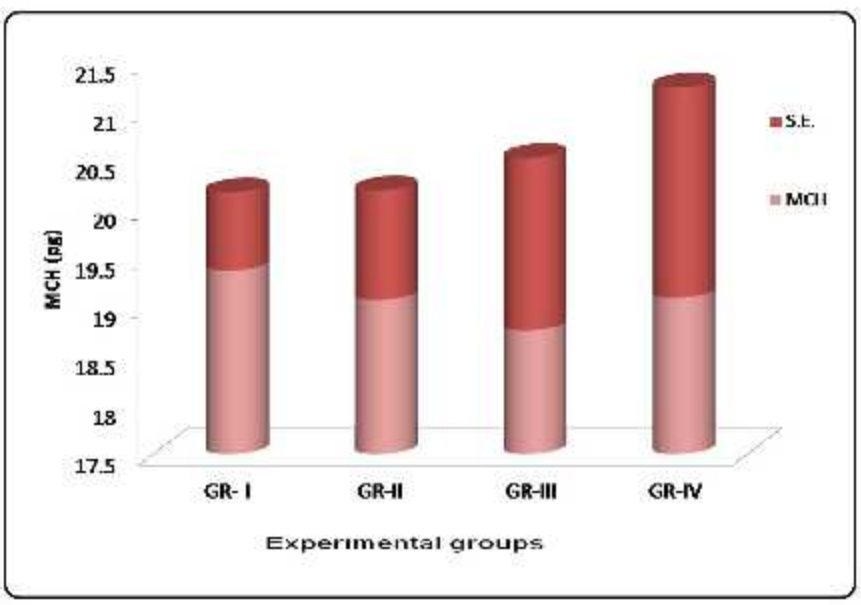


Fig.23

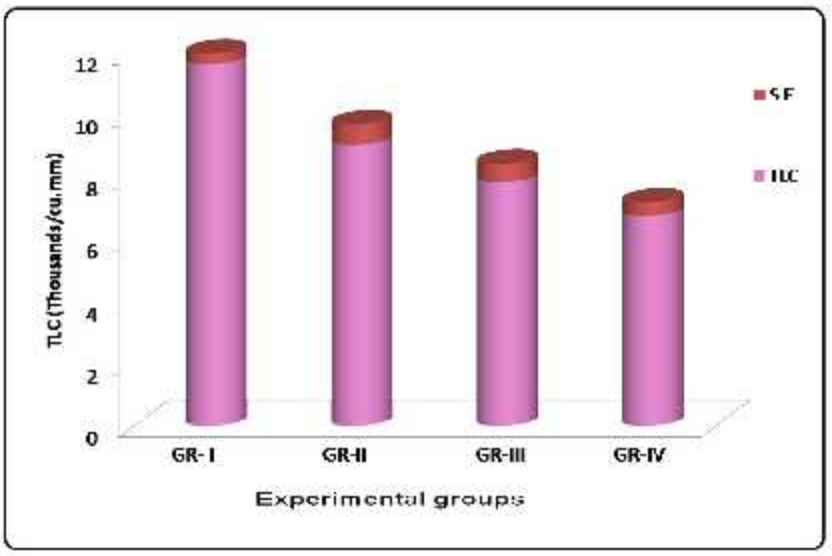


Fig. 24

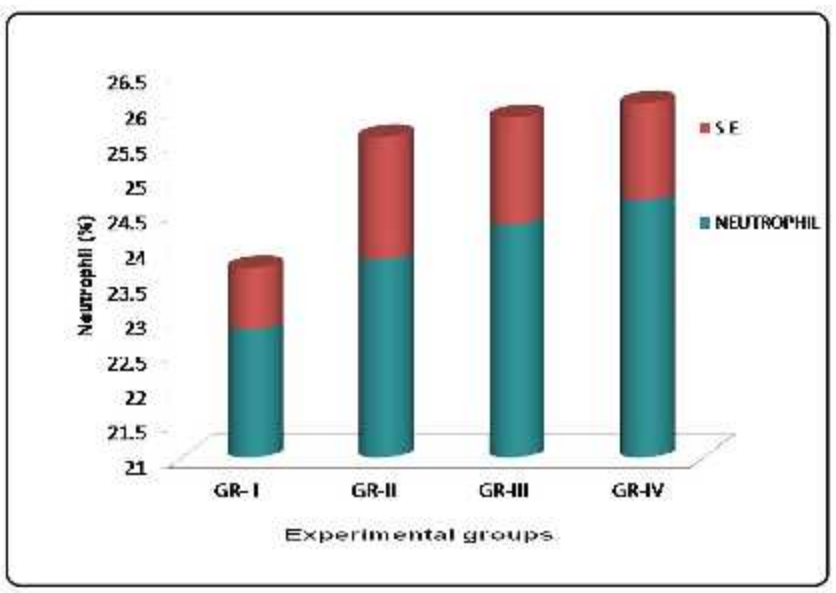
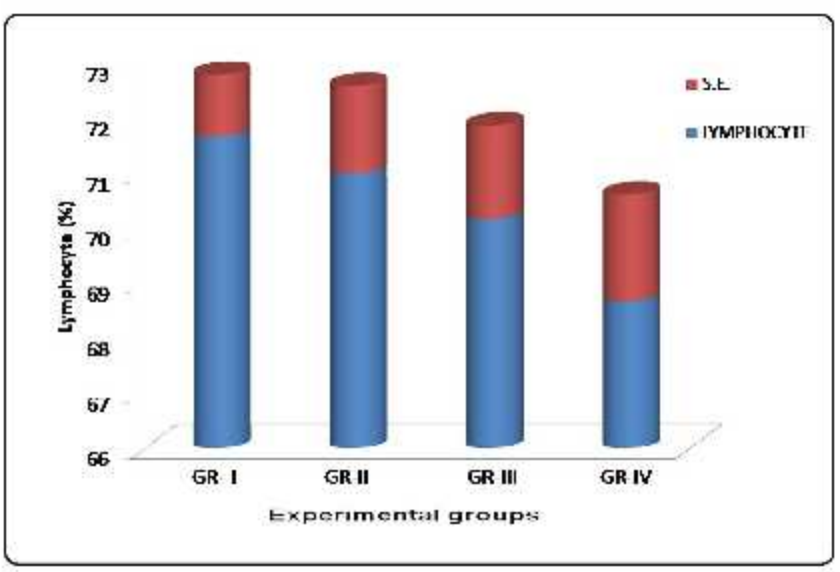


Fig.25



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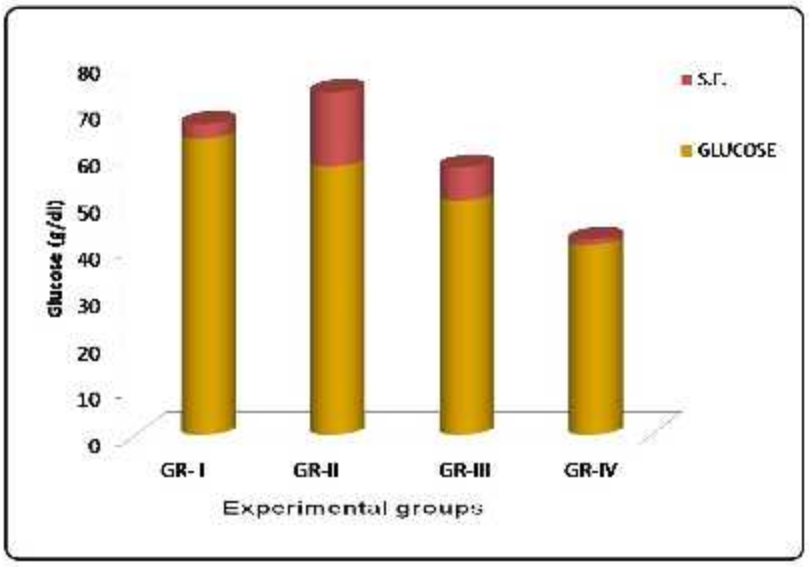
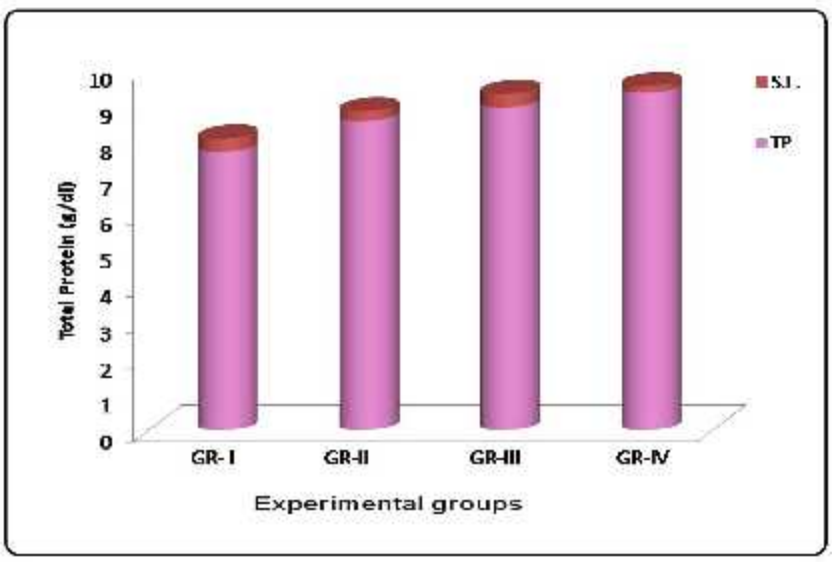


Fig. 30



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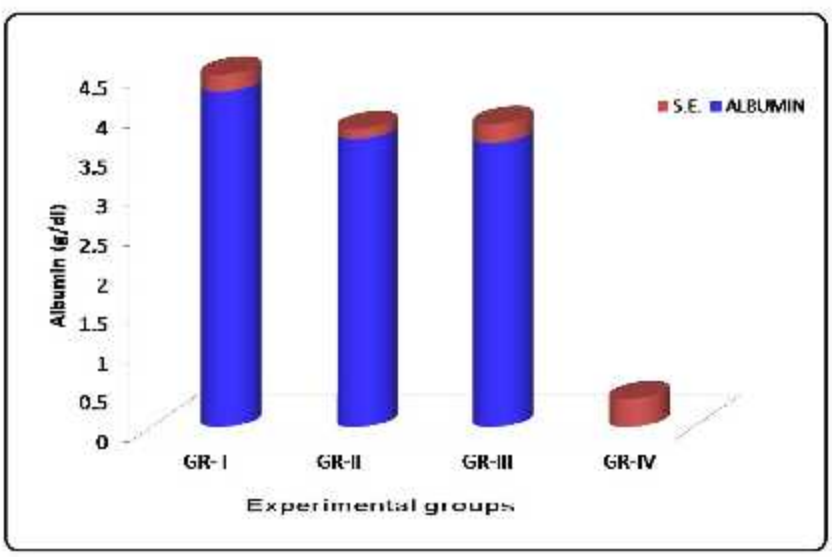

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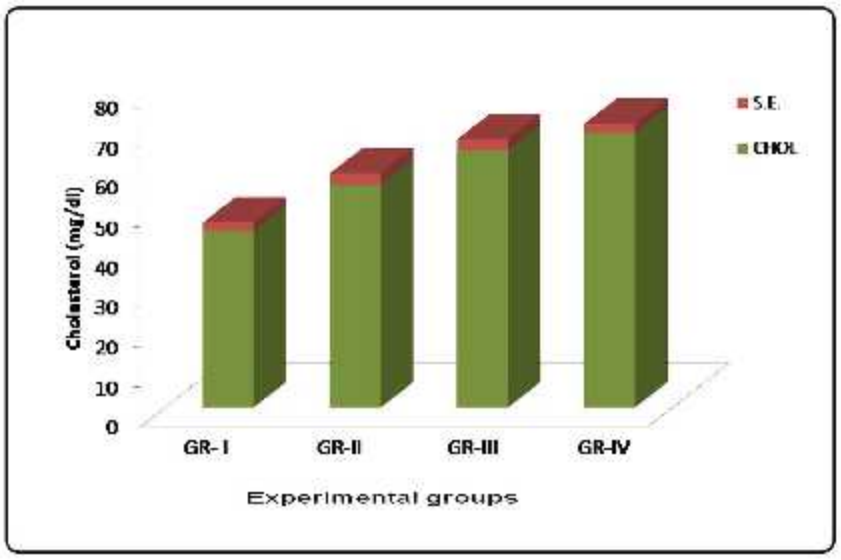

80
70
60
50
40
30
20
10
0
Cholesterol (mg/dl)
S.E.
CHOL
GR-I
GR-II
GR-III
GR-IV
Experimental groups

Fig. 36

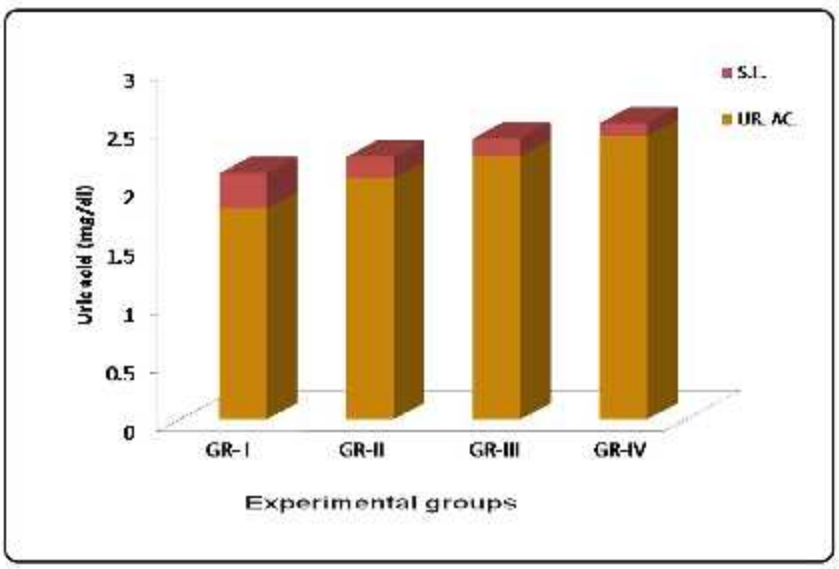

3
2.5
2
1.5
1
0.5
0
Uric acid (mg/dl)
S.E.
UR. AC.
GR-I
GR-II
GR-III
GR-IV
Experimental groups

Fig. 37

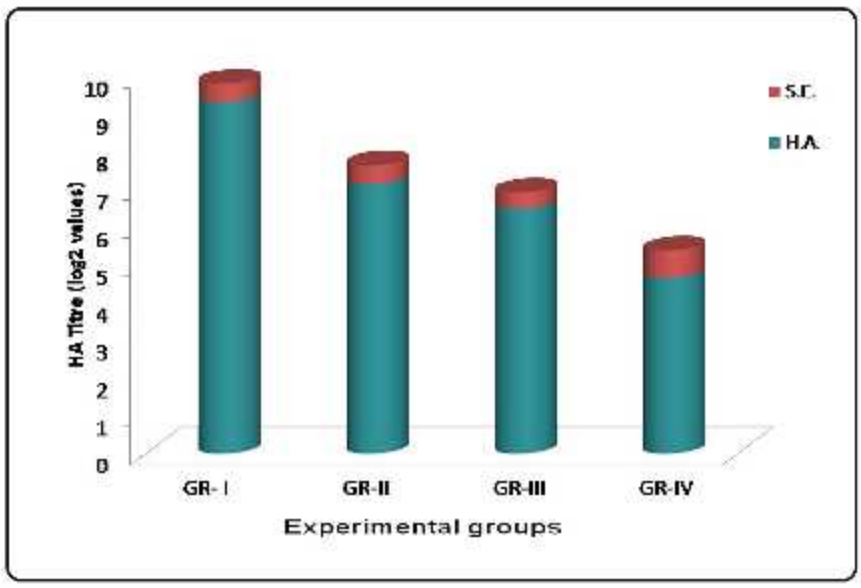

10
9
8
7
6
5
4
3
2
1
0
HA Titre (log2 values)
S.C.
H.A.
GR- I
GR-II
GR-III
GR-IV
Experimental groups

Fig. 38

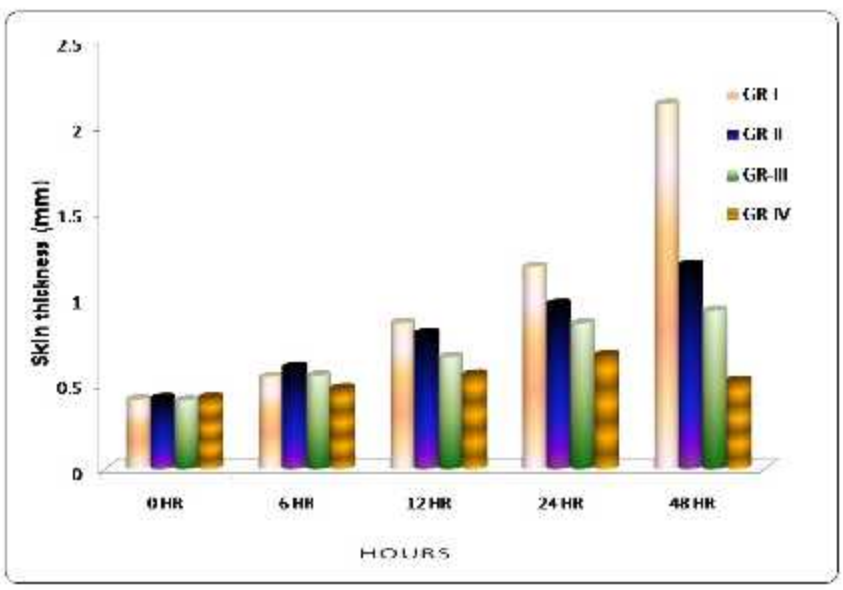

2.5
2
1.5
1
0.5
0
Skin thickness (mm)
GR I
GR II
GR-III
GR IV
0 HR
6 HR
12 HR
24 HR
48 HR
HOURS

Fig. 44

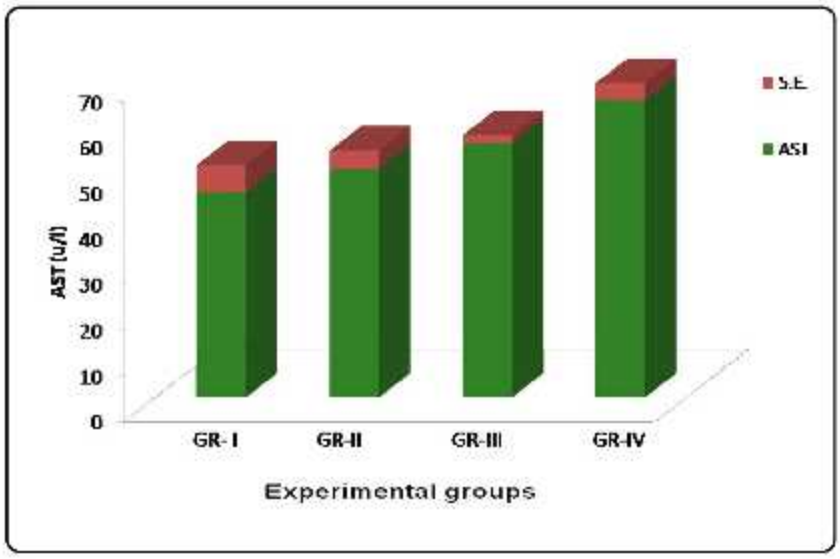


Fig. 27

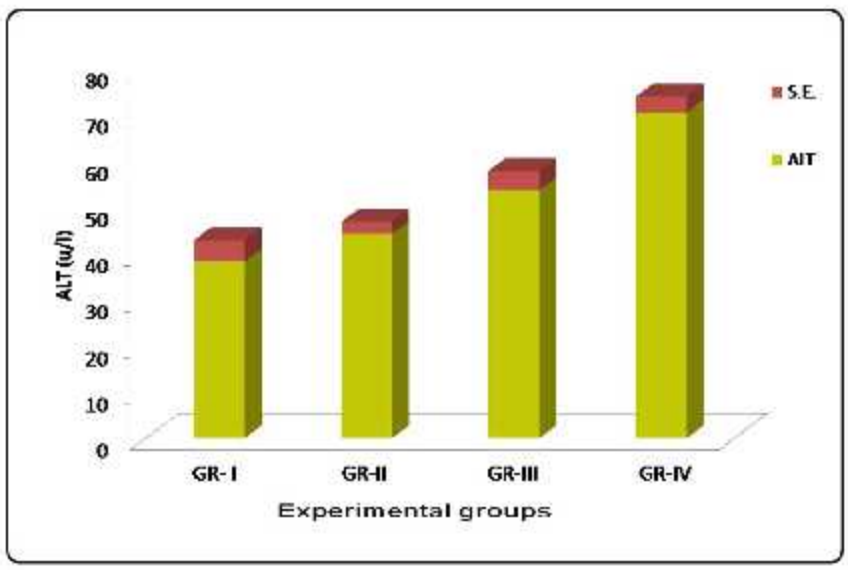


Fig.28

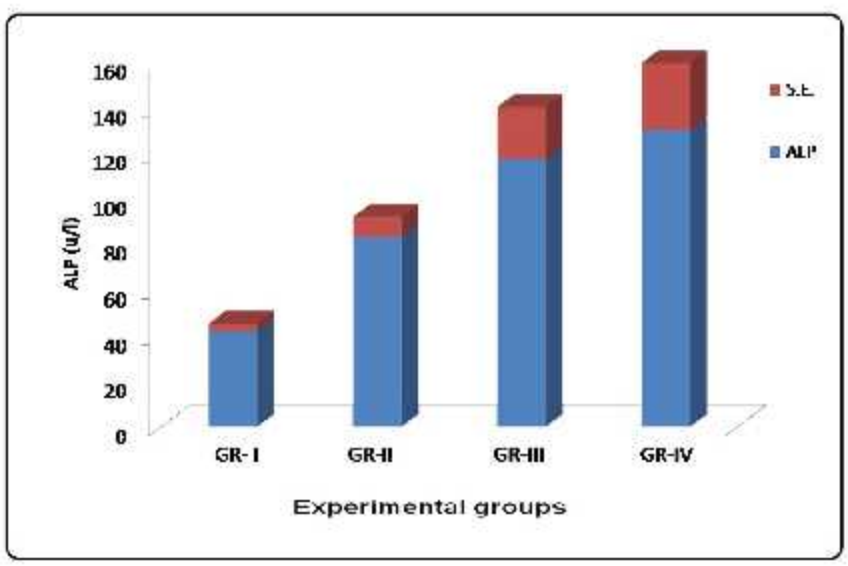


Fig.29

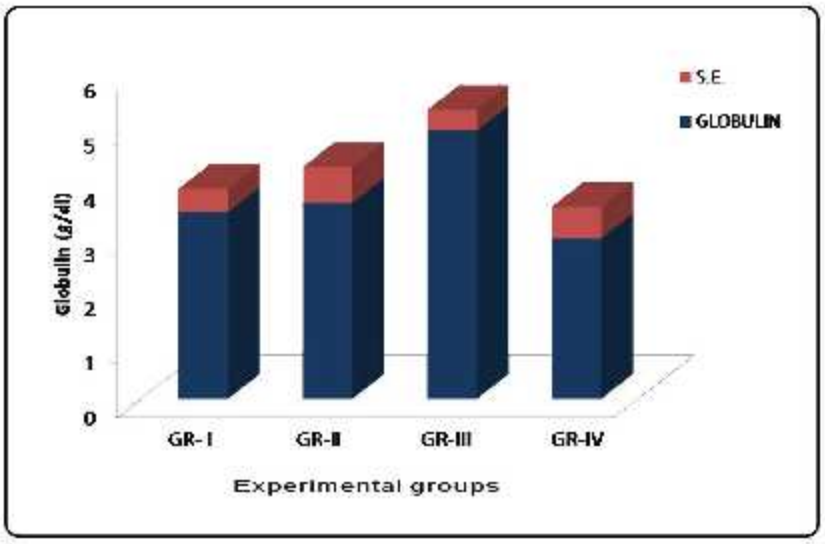


Fig. 33

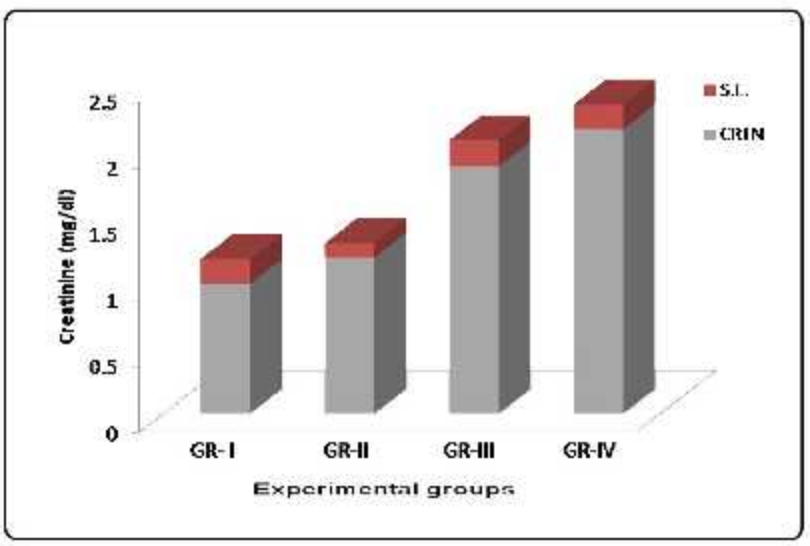


Fig. 34

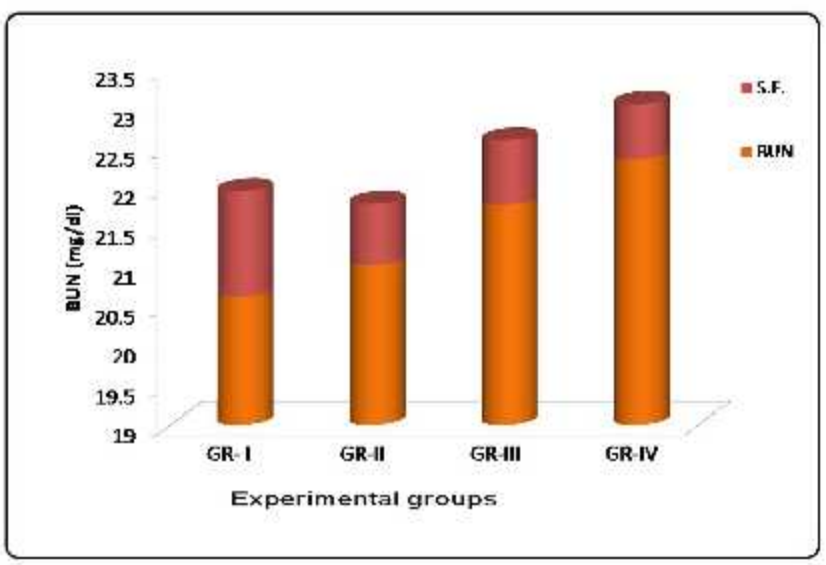


Fig.35

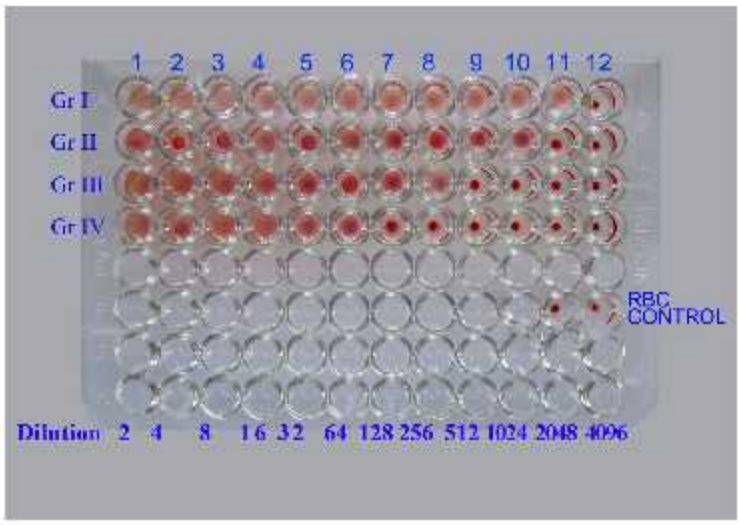


Fig. 39

Fig. 40

Fig. 41

Fig. 42

Fig. 43

The status of the humoral immunity of the rats exposed to sodium benzoate toxicity against sheep red blood cells (SRBC) was assessed by micro HA test. The HA titer of the rats of all the sodium benzoate treated groups decreased significantly ($P \leq 0.01$) as compared to the rats of control group **(Fig. 39)**. The present findings are in accordance with the findings of Priya *et al.* (2008) who reported significant ($P \leq 0.05$) decrease in the humoral immunity to sheep RBC at 50 mg sodium benzoate/ kg body weight in rats.

There was progressive decrease in HA titer with increasing dose of sodium benzoate indicating adverse effect of sodium benzoate on humoral immunity. Also, there were progressive decline levels of mean plasma globulin. The level of serum antibodies against the antigen is the conventional index of humoral immunity, which could be measured accurately by HA titer (Gatne *et al.*, 2006). The decline in HA titre to SRBC in the present study could be due to effect of sodium benzoate in the inhibition of degradation of antigen by the reticuloendothelial system (cited from Ghosh and Chauhan, 1991) and inhibition of immunoglobulin synthesis (Clifford and Rees, 1967 and La Farge and Frayssinet, 1970). From the present study it is logical to expect that short term exposure of sodium benzoate exhibits leucocytopaenia and hypoglobulinemia resulting in reduced humoral immune response and increasing susceptibility of rats to various infections.

4.2.6.2 Cell mediated immune response

The cell mediated immune response in rats was assessed by chemical contact sensitization with DNFB test. Application of the challenge dose of

DNFB caused erythema, oedema, vesiculation and scabbing **(Fig. 40, Fig. 41, Fig. 42** and **Fig. 43)**. The skin reaction was more marked at 24 h of post challenge. The mean skin thickness in rats of different groups recorded has been given in **Table 9** and depicted in **Fig. 44**.

There was significant ($P \leq 0.01$) increase in the ear thickness of control rats compared with the sodium benzoate treated rats at 24 and 48 h post challenge. This indicates depression of cellular immunity due to sodium benzoate toxicity. Similarly, Priya *et al*. (2008) observed significant decrease in the cell mediated immune response to stimulation indices of spleenocytes in rats treated with sodium benzoate @ 25 and 50 mg/kg body weight. Similar result of depressed CMI have been reported in mice and goat treated with cypermethrin (Tamang et al., 1988) and in crossbred calves treated with cypermethrin (Patel *et al*., 1996). However, a stimulatory effect on the immune response in sheep treated with phosalone was observed (Dimov and Simenov, 1995).

Immunosuppressive effect observed in the present study could possibly be due to depletion of lymphoid cells in the spleen. Thus the depression of cellular immunity due to sodium benzoate dosing might be the result of a possible cytotoxic effect of sodium benzoate on T-lymphocytes. Continuous exposure to sodium benzoate may affect the immune system adversely making them susceptible to various diseases leading to mortality.

4.2.7 Pathology of sodium benzoate toxicity

Detailed post-mortem examinations of all the rats of different groups were performed on day 29th of the experiment. For the rats which died during experiment, post-mortem was performed immediately after death. Different visceral organs in the control group (group I) did not exhibit any lesions of pathological significance.

4.2.7.1 Liver

Gross pathology

Sodium benzoate treated groups revealed moderate to severe congestion. Liver of rats of group III were found to be pale yellow in colour **(Fig. 45)** with rounded edges indicating fatty changes. Liver of rats of group IV showed slight enlargement and congestion with petechial hemorrhages **(Fig. 46)**. Report on histopathology in sodium benzoate toxicity in rats is scanty in the available literature. However, Piramanayagam and Manohar (2002) reported mottling of liver in gross changes induced by malathion toxicity in rats. Bhelonde (2001) documented enlargement of liver in rats (@ 5.916 mg/kg body weight) in fenpropathrin toxicity.

Histopathology

Microscopical feature of liver intoxicated with sodium benzoate showed severity of changes in dose dependent manner. Mild degenerative and necrotic changes were seen in liver of rats of group II. The intense degenerative changes with hydropic degeneration were observed in liver of rats of group III. Also, there was congestion of sinusoids, severe haemorrhages **(Fig. 47)** along with presence

Table 9. DNFB response (Mean increase in ear thickness in mm) of rats exposed to subacute sodium benzoate toxicity (Left side served as vehicle control and right side treated with the DNFB) [n = 6]

Groups	Ear side	Before sensitization	After challenge with DNFB at different time interval (h)			
			6	12	24	48
Gr I	Left	0.405±0.015[a]	0.440±0.013[a]	0.437±0.011[a]	0.453±0.11[a]	0.455±0.006[a]
	Right	0.403±0.011[a]	0.535±0.014[a]	0.850±0.029[a]	1.178±0.031[a]	2.128±0.042[a]
Gr II	Left	0.388±0.008[a]	0430±0.007[a]	0.423±0.009[a]	0.422±0.009[b]	0.425±0.012[b]
	Right	0.408±0.006[a]	0.590±0.028[a]	0.787±0.047[a]	0.958±0.034[b]	1.188±0.035[b]
Gr III	Left	0.397±0.014[a]	0.428±0.013[a]	0.422±0.012[a]	0.430±0.121[ab]	0.438±0.010[ab]
	Right	0.398±0.006[a]	0.545±0.021[a]	0.652±0.021[b **]	0.848±0.034[c **]	0.923±0.029[c **]
Gr IV	Left	0.400±0.009[a]	0.412±0.007[a]	0.427±0.007[a]	0. 415±0.008[b]	0.417±0.006[b]
	Right	0.412±0.006[a]	0.467±0.010[b]	0. 548±0.017[c **]	0.662±0.032[d **]	0.512±0.035[d **]

Superscript may read column wise for mean comparison.
Similar superscript showing means do not differ significantly. (*P≤0.05) (**P≤ 0.01)

of thrombus in hepatic vein of liver of group III **(Fig. 48)**. The liver of rats of group IV showed severe degenerative and necrotic changes (pyknotic nuclei) **(Fig. 49)**. There was focal necrosis leading to lysis of hepatocytes **(Fig. 50)**. High vacuolization and glassy cytoplasm in rats and mice were observed in sodium benzoate intoxication (Fujitani, 1993). Similar findings were obtained in the liver of rats treated with BHT (Mcfarlene *et al.*, 1997), sodium benzoate and benzoic acid in mice (Kaboglu and Aktac, 2002 and Aktac *et al*., 2002).

In the present study, congestion and reduction in blood circulation might have caused anoxia, which resulted in degenetative and necrotic changes in liver (Mondal, 2007). The liver changes comprised of mild haemorrhages and fatty changes due to decomposition and metabolism of the sodium benzoate in to hippuric acid in liver. The severe degenerative changes would have possibly interfered with transportation or metabolism of lipid leading to accumulation of lipid in liver (Sawale, 2002). Also, increased serum activity of AST and ALT of rats intoxicated with sodium benzoate support the histological findings.

In the liver, sodium benzoate treated rats revealed moderate to severe toxic hepatosis. Sodium benzoate could possibly be a hepatotoxin that alters mitochondrial and microsomal functions.

4.2.7.2 Lungs

Gross pathology

At necropsy, enlargement and ecchymotic haemorrhages in lung were found in rats of group IV receiving sodium benzoate @ 400 mg/kg body weight **(Fig. 51)**. Some rats revealed highly congested lungs with linear and petechial

haemorrhages **(Fig. 52)**, consolidation and pneumonic patches in rats of group IV. Rats of group III showed slight enlargement, congestion **(Fig. 53)** and haemorrhages in lungs of rats of group III. The rats of group I (control) and group II did not reveal any gross alterations in any of the organs.

Bhelonde (2001) observed no significant alterations in any groups except slight enlargement of the liver in rats of group receiving high dose of fenpropathrin. Manna *et al.* (2004) found haemorrhages in lung of rat on gross examination in α- cypermethrin toxicity.

Histopathology

The effects of sodium benzoate in lungs of albino rats were dose dependent. Lungs of rats of group II showed mild congestion. There were mild congestion and haemorrhages in lungs of rat of group III **(Fig. 54)**. Bronchi of some rats were filled with RBCs. Also, some bronchioles were infiltrated with mononuclear cells and serous exudates in lungs of rat of group III **(Fig. 55)**. There was moderate degree of edema in lung of rats of group IV **(Fig. 56)**. Some rats showed severe hemorrhages in lungs of group IV **(Fig. 57)**.

Almost similar finding was reported by Erdogan *et al*. (2006) in deltamethrin toxicity and Bhelonde (2001) in subchronic fenpropathrin toxicity in rats, while Mani *et al*. (2001) found presence of edematous fluid in alveolar lumen along with other similar type of findings. Amaravathi *et al*. (2009) reported area of emphysema and thickened alveolar septa due to edema, infiltration of mononuclear cells (MNCs) and RBCs. Hyalinised blood vesels and giant cells in alveolar lumen were observed prominently in higher doses of fenvelarate treated

rats. However, microscopic lesions of the lungs in paraquat toxicity in calves revealed varying degree of emphysema, moderate congestion and haemorrhages around the pulmonary vessels and interstitial spaces (Tamuli *et al.*, 2005). Haemorrrhages and thickened interalveolar septae with infiltration of mononuclear cells in lungs were reported after daily oral administration of α-cypermethrin in rats for 30 days (Manna *et al.*, 2004a).

4.2.7.3 Kidney

Gross pathology

There were no obvious gross lesions noted in the kidneys of rat of group I and II. Increase in weight of the kidneys was found in the rats of group III and IV. Also, mild congestion was observed in the kidneys of rats of group III while kidneys of rat of group IV showed severe congestion **(Fig. 58)**. Some of the rats also revealed pale kidneys.

Similar findings were observed by Huey-Jen *et al*., (2007), who reported nephrotoxic effect of sodium benzoate on zebrafish larvae. Bhagat *et al.*, (2009) reported petechae and echymotic kidneys in broiler experimentally induced with chlorpyriphos toxicity. However, Tamuli *et al.* (2005) observed no gross lesions in the kidneys of the calves, except perirenal gelatinization of fat during paraquat intoxication.

Histopathology

Histopathological sections of kidney revealed mild congestion in group II rats. In the rats of group III, moderate degree of degenerative and necrotic changes in proximal and distal convoluted tubules were noted **(Fig. 59)**. Also, there were

congestion and haemorrhages in kidney of rats of group III. Rats of group IV revealed severe degenerative and necrotic changes in proximal convulated tubules of kidney. There were necrosis of glomerular tuft **(Fig. 60)**, haemorrhages and severe congestion of blood vessels with presence of albuminous cast in some of the PCT and DCT of kidneys of rats of group IV (**Fig. 61** and **Fig. 62**).

No histopathological lesions except mild congestion have been found in kidneys of rats administered with imidacloprid at 20mg/kg (Premlata, 2001). Severe congestion of the blood vessels, desquamation or coagulative necrosis of the epithelial cells of the tubules and proliferation of the endothelial cells of the glomeruli were seen in the kidney of goats due to cypermethrin intoxication (Tamang, 1987). Jana *et al.,* (2009) reported extensive changes in kidney involving both glomeruli and tubules with abundant deposition of urate crystals in the form of spongy balls and presence of casts in some of the dilated tubules in kidney of broiler chickens affected with gout. Congestion and extravasation of erythrocytes in cortex and medulla, along with cloudy swelling to necrotic changes in lining epithelium of PCT with or without proliferation of convulated tubules of kidney of rats were induced with lead toxicity (Satish *et al.,* 2009). The kidney revealed focal tubular necrosis, shrinkage of glomerular tuft with widening of urine formation spaces in glomeruli and presence of granular cast in tubular epithelium of rats intoxicated with atrazine (Vijay *et al*., 2009).

In the present study, the result obtained from the experiment, sodium benzoate has been found nephrotoxic. Thus, it appears that sodium benzoate might have a direct toxic effect and or result of anoxia due to congestion.

Fig. 45

Fig. 46

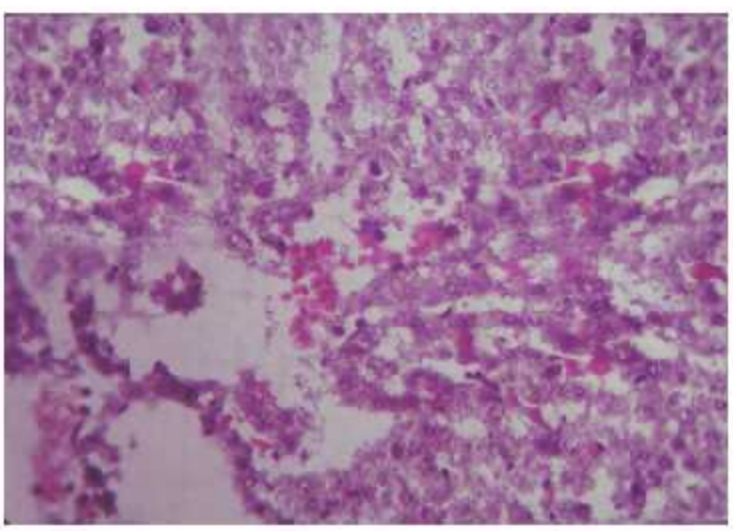


Fig. 47

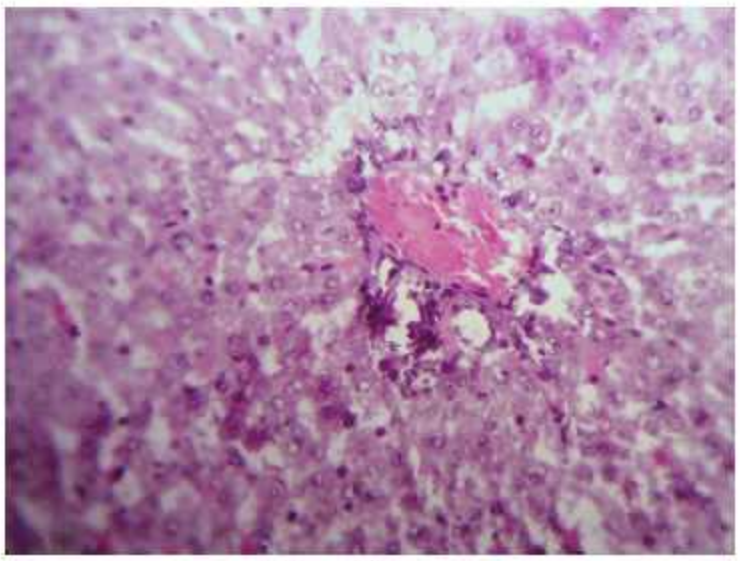


Fig. 48

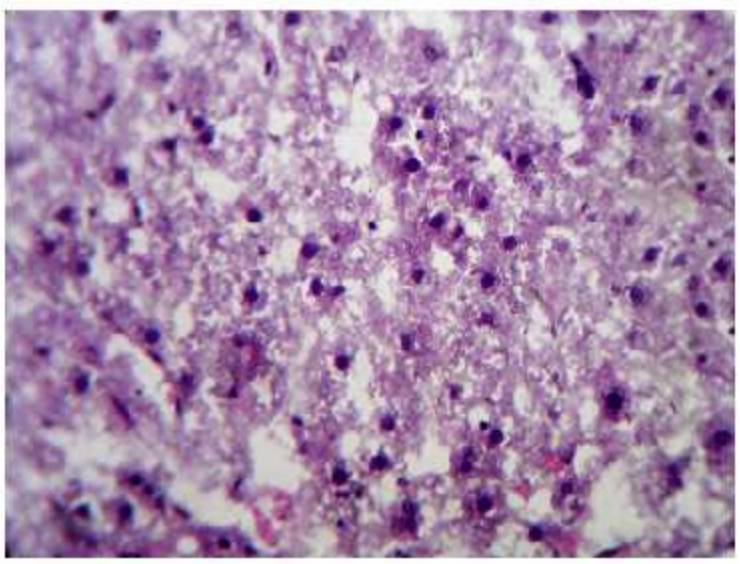


Fig. 49

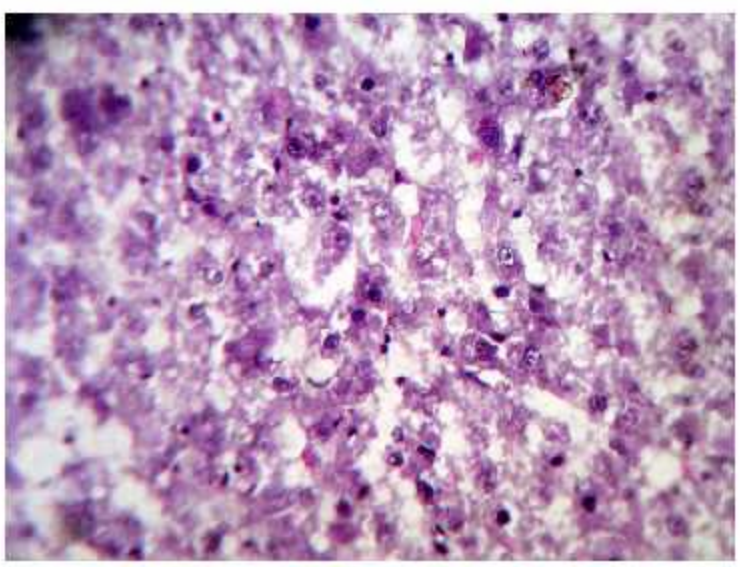


Fig. 50

Fig. 51

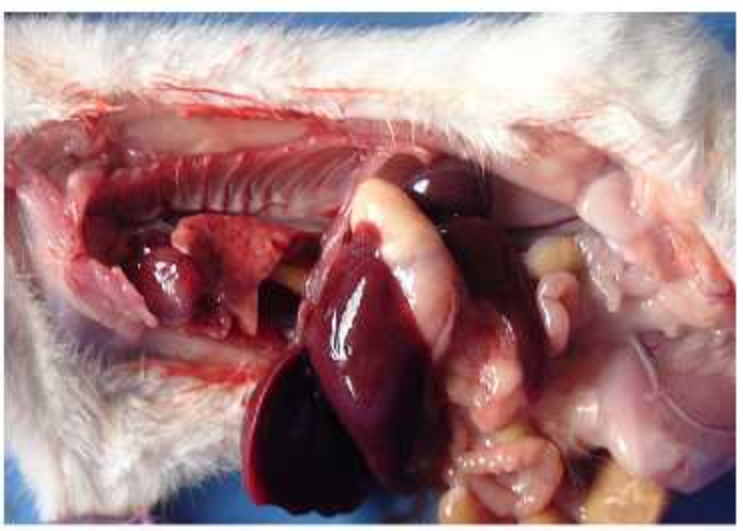


Fig. 52

Fig. 53

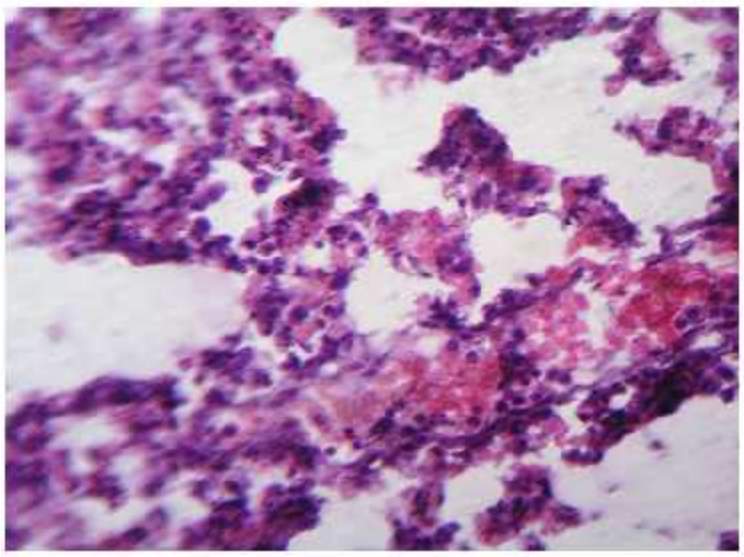


Fig. 54

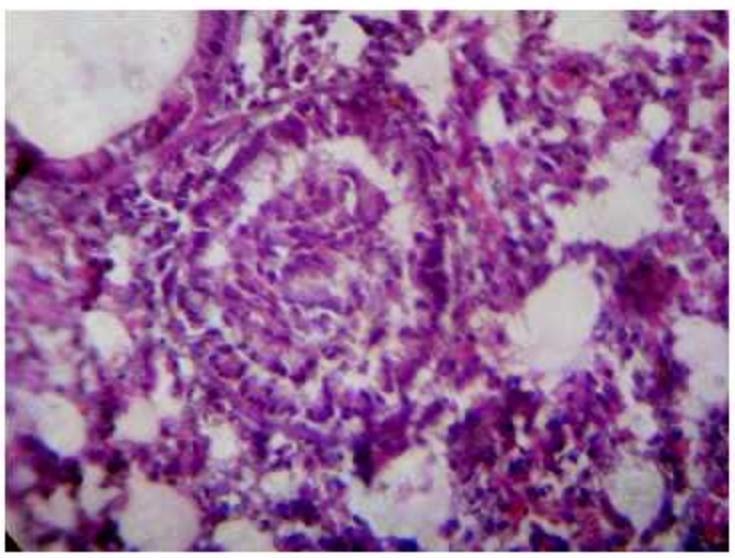


Fig. 55

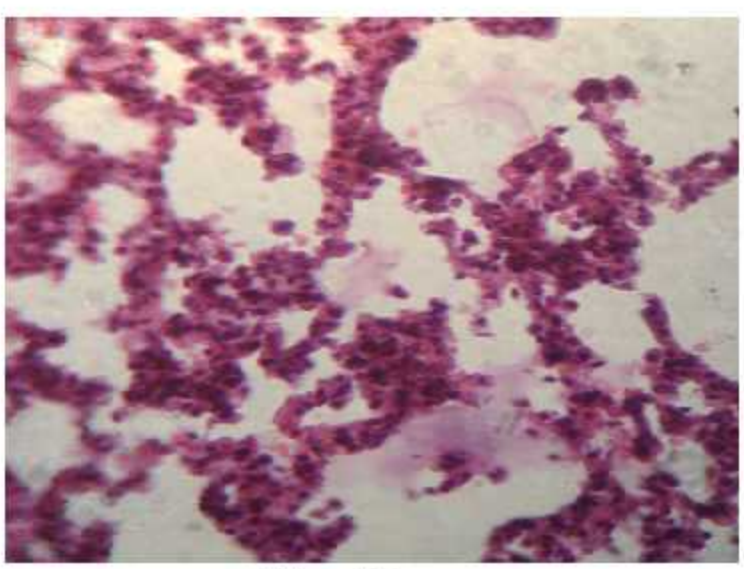


Fig. 56

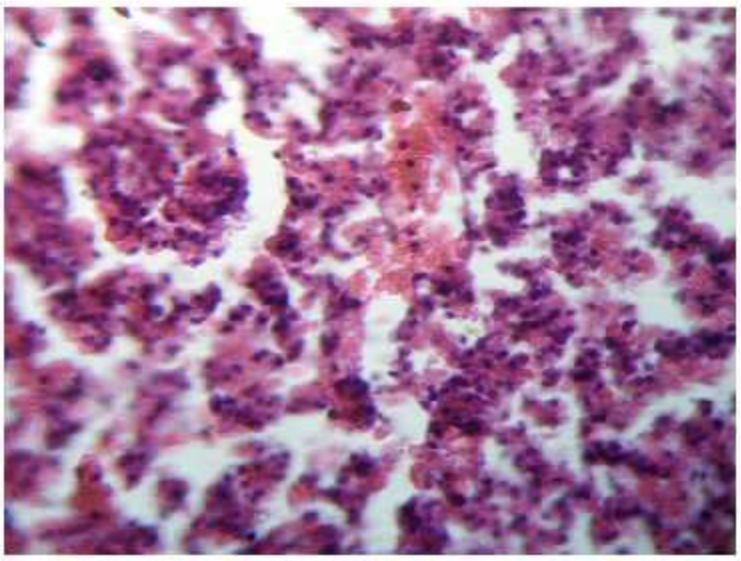


Fig. 57

Fig. 58

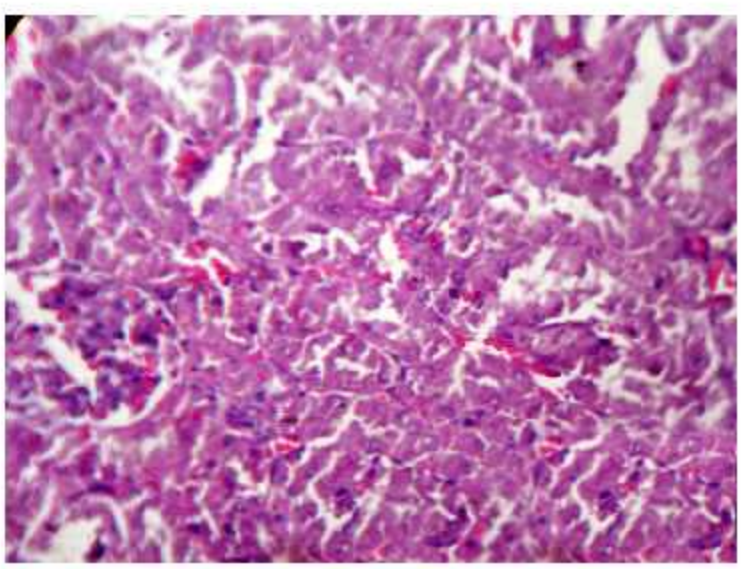
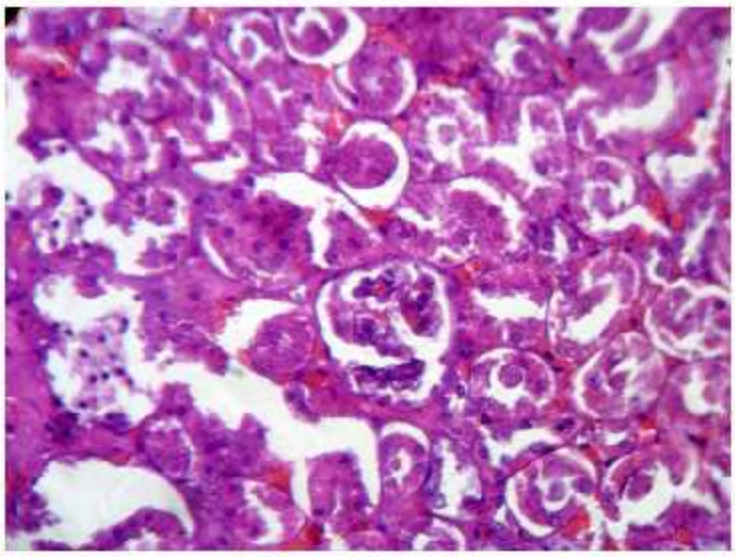


Fig. 59



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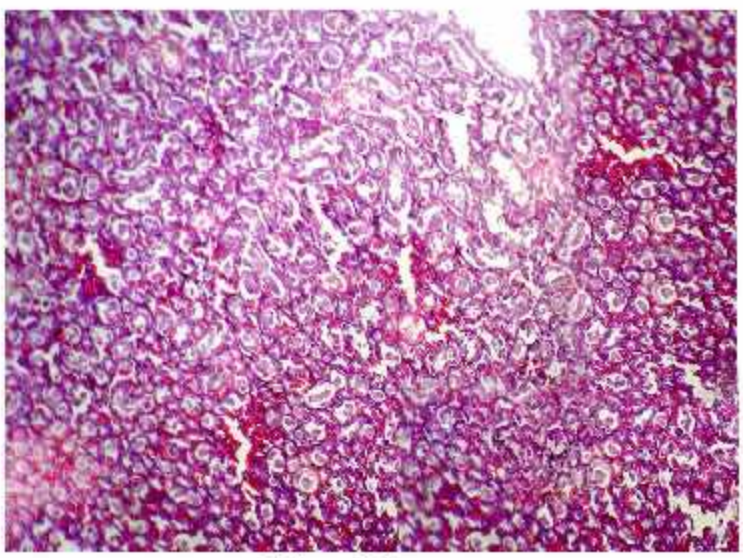


Fig. 61

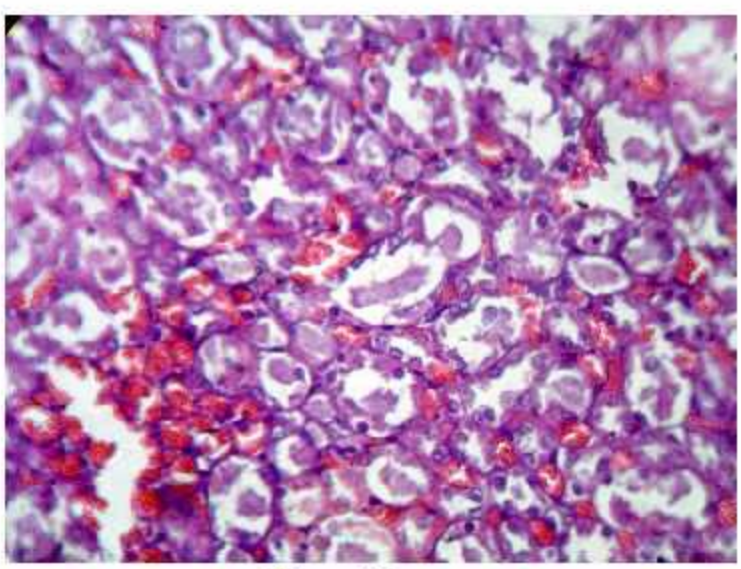


Fig. 62

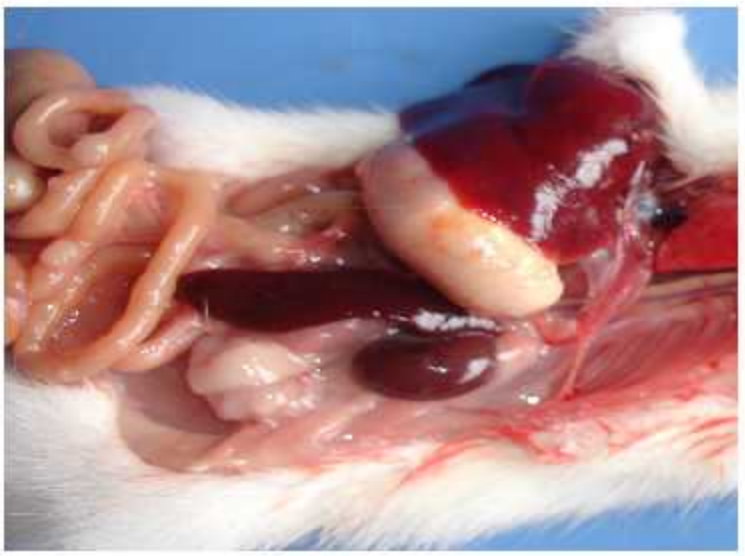


Fig. 63

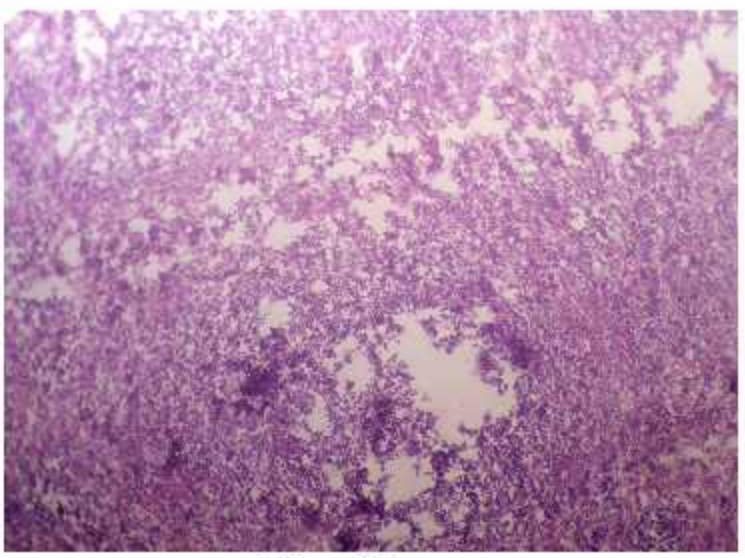
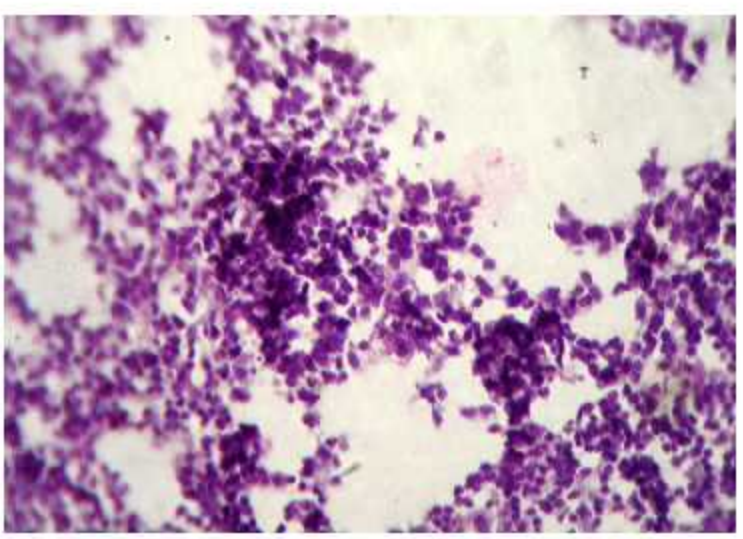


Fig. 64



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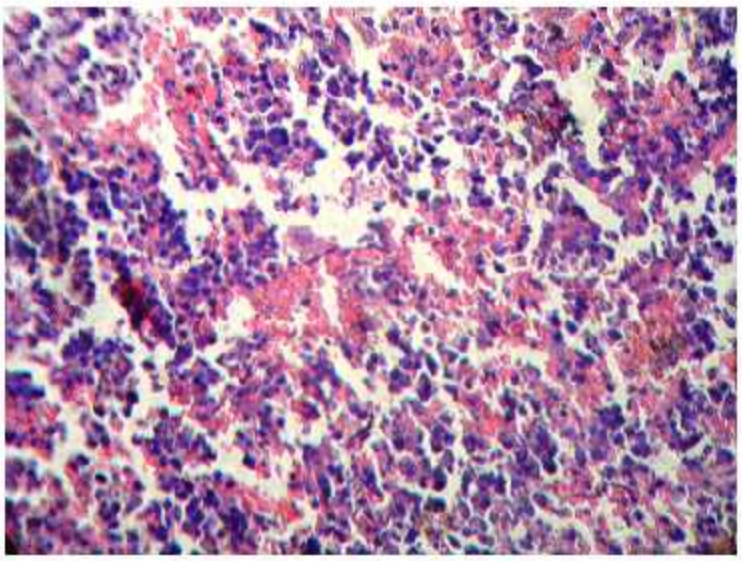

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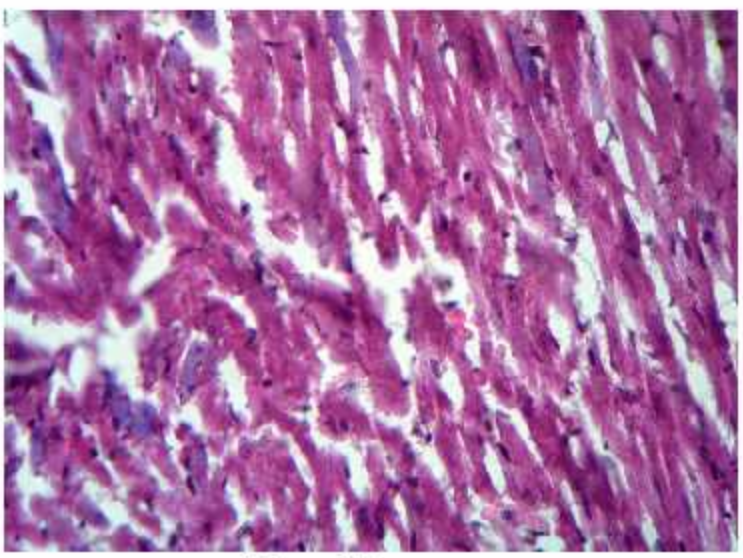

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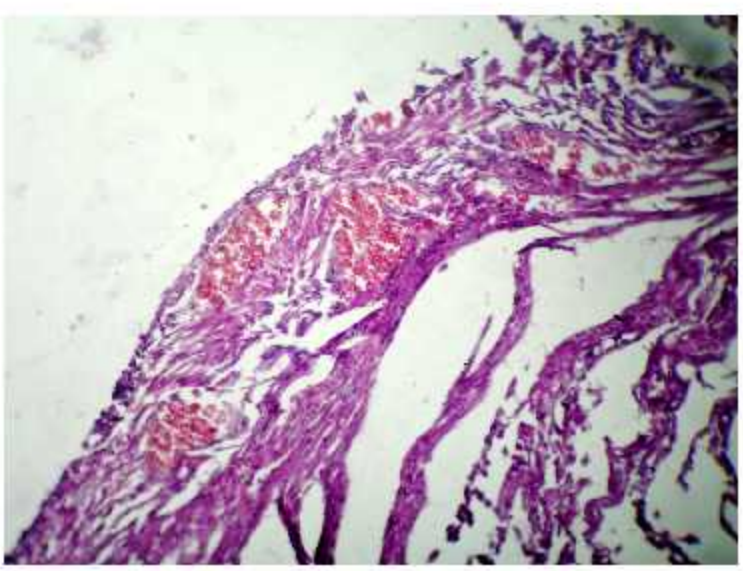

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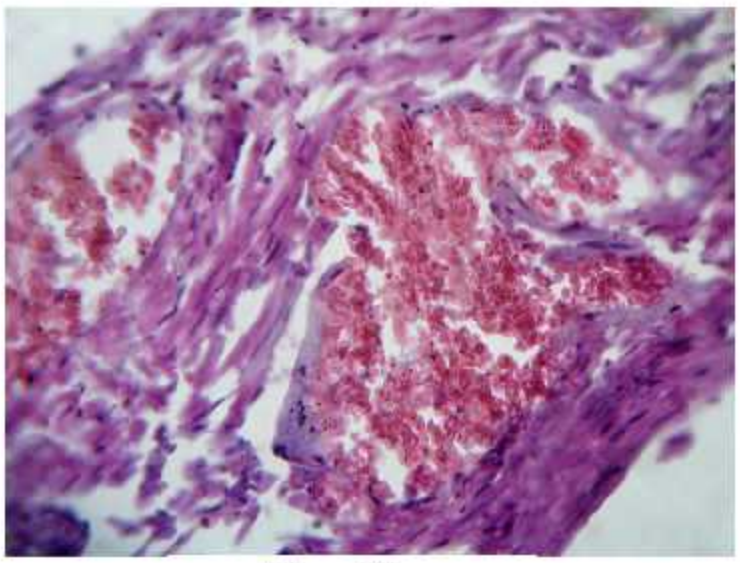

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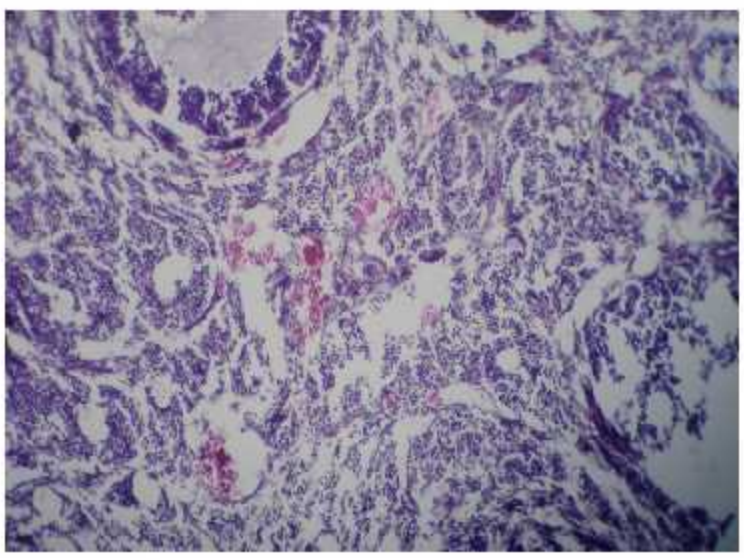

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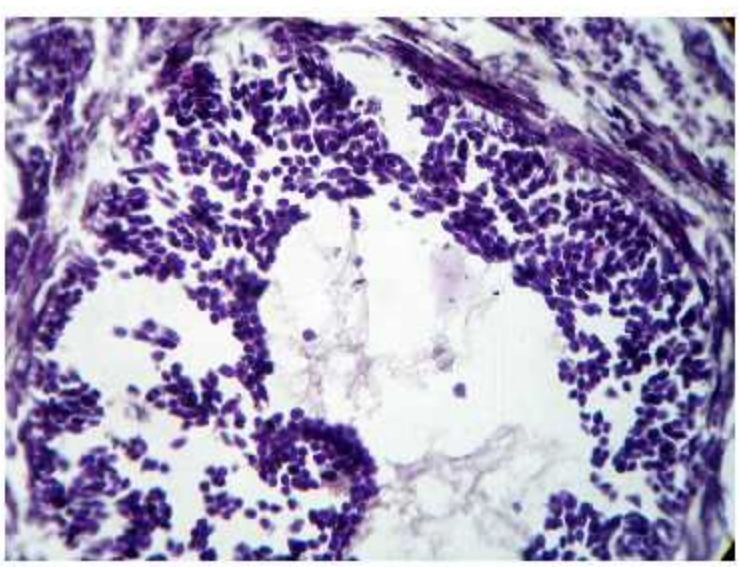

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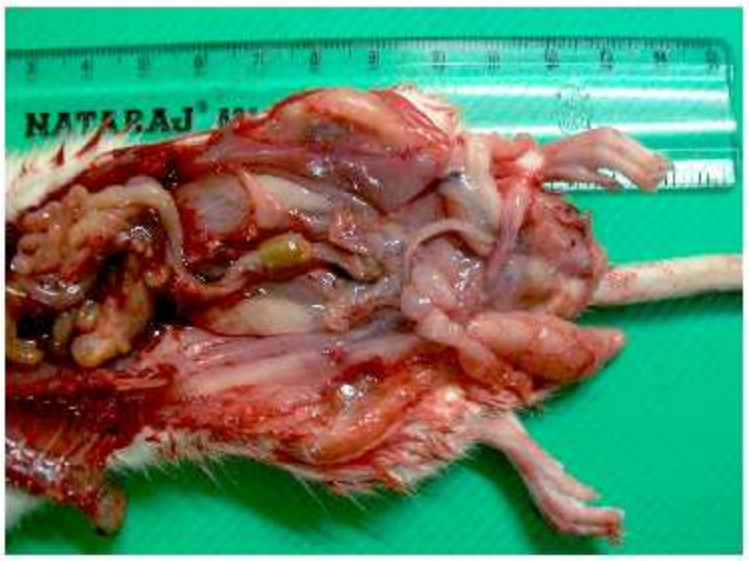

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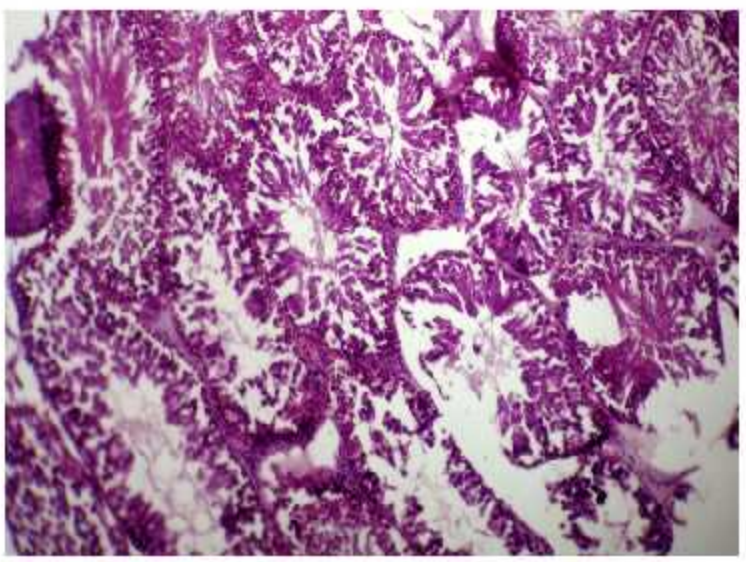

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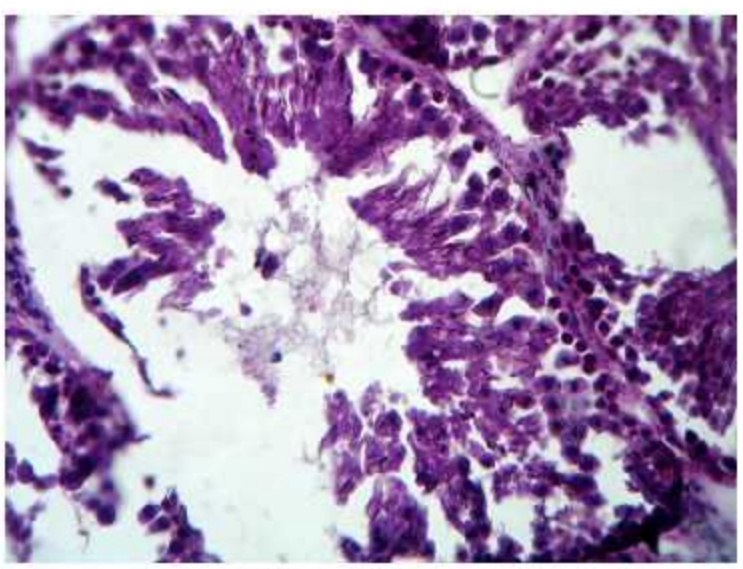

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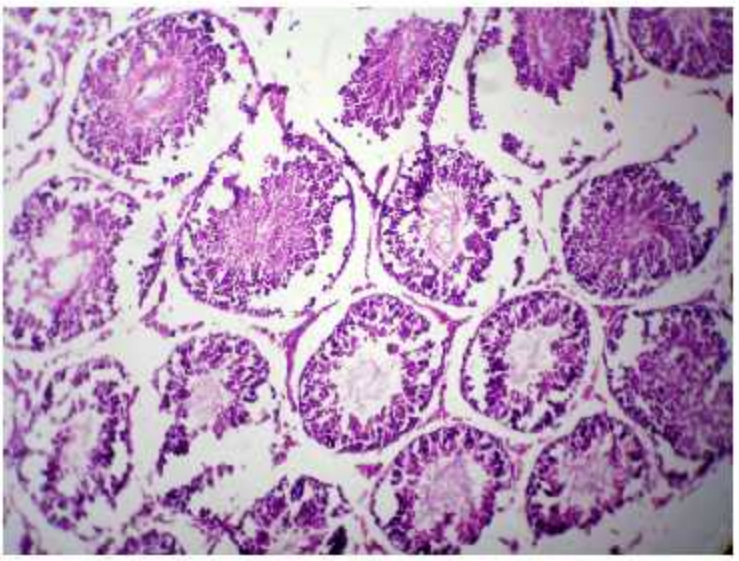

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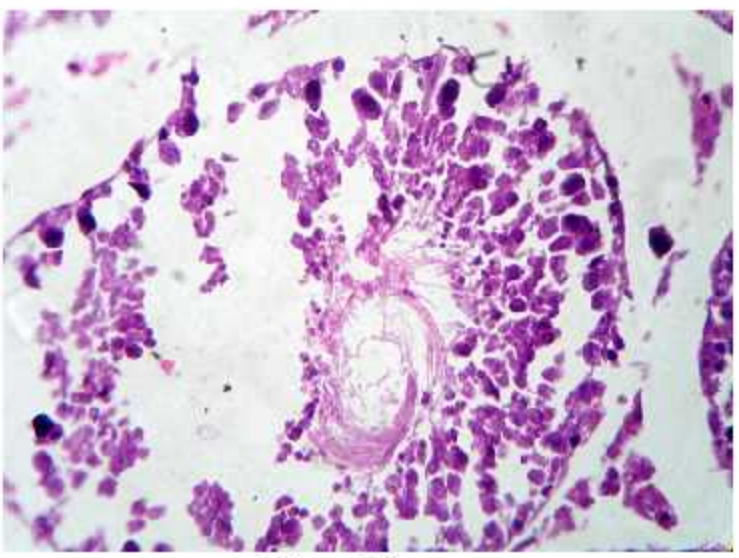

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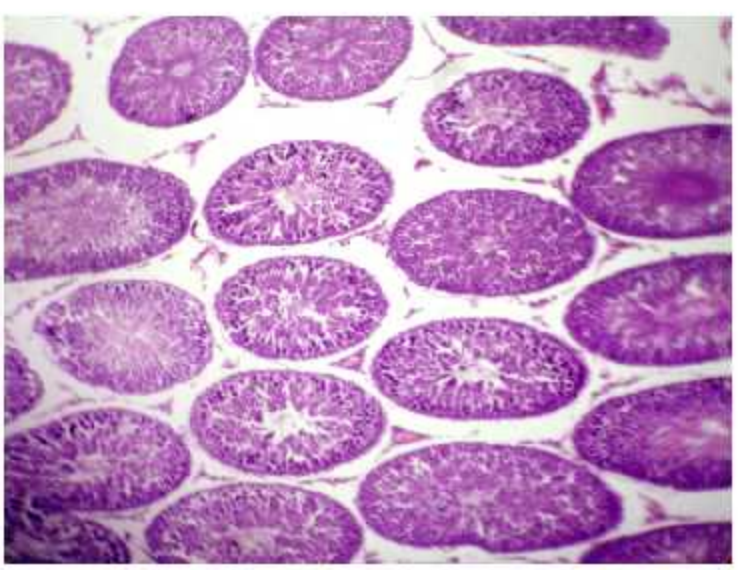

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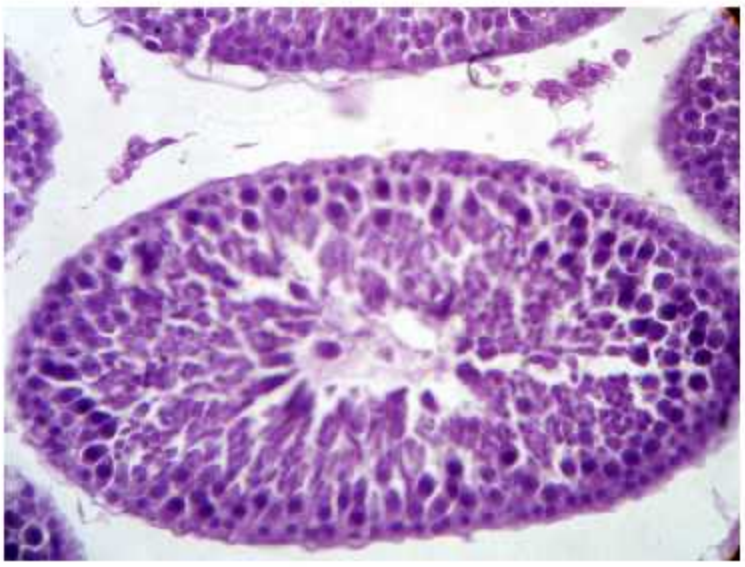

Fig. 78

Fig. 79

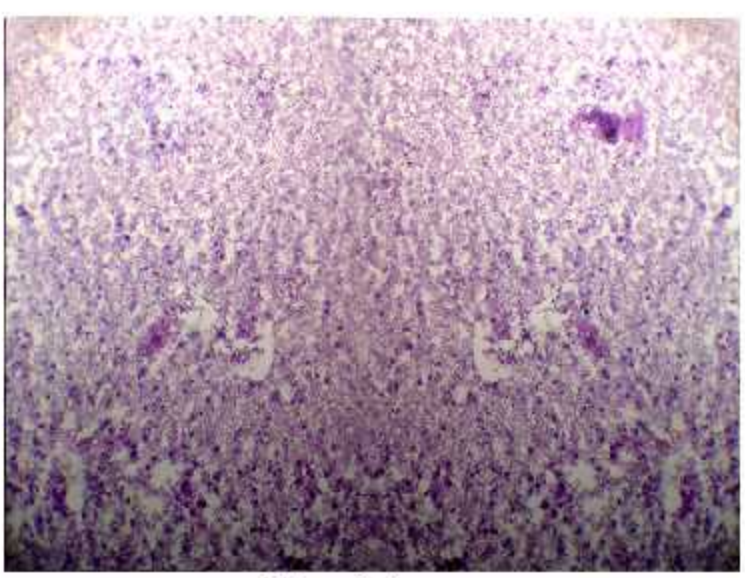

Fig. 80

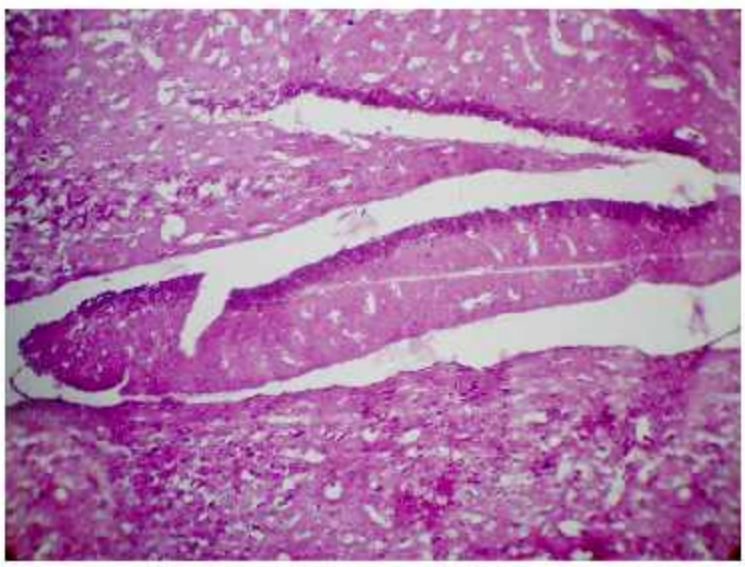

Fig. 81

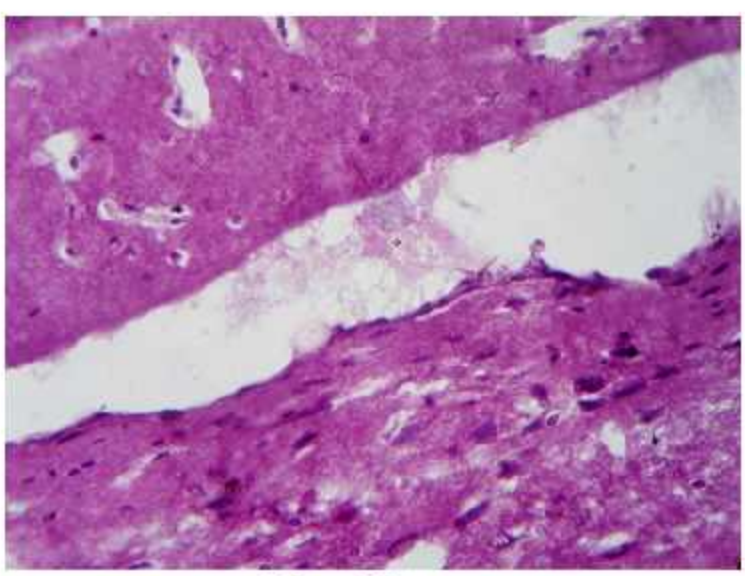

Fig. 82

4.2.7.4 Spleen

Gross pathology

There were no changes recorded at necropsy in spleen of rats of group I and group II. A slight increase in size of spleen in the rats of group III and group IV was observed along with severe congestion **(Fig. 63)**.

Histopathology

Histopathological changes in the spleen revealed severe depletion of lymphocytes in the Malpighian corpuscles (**Fig. 64** and **Fig. 65**) and subsequent necrosis of the white pulp of the spleen of rats of group IV. The size of Malpighian corpuscles also appeared to be smaller than normal. Haemorrhages were also noticed at some places in spleen of group IV rats. However, red pulp and trabaeculae of the spleen did not reveal any significant changes. Moderate depletion of the lymphocytes in the white pulp of the spleen was observed in the spleen of mid dose group (group III). Severe congestion was observed in the spleen of rats of group II **(Fig. 66)**. However, no noticeable changes were observed in spleen of rats of control group.

Our findings in the spleen of sodium benzoate treated rats are in agreement with the findings of Mishra and Kuswah (2007) who observed vacuolation and necrosis of splenic cells and marked haemorrhages in the spleen of rats in toxicity of monocroptophos. Cha *et al.* (2000) observed depletion of spleen lymphocytes in the periarteriolar lymphoid sheath and marginal zone in white pulp. Such retrogressive changes of the lymphoid cell population have also been observed in rabbits (Desai *et al.*, 1986) and rats (Ragothaman, 1991) due to cypermethrin

Appendix

Alsever's solution:

For 100 ml:

Dextrose	-	2.05 g
Sod. Citrate (dihydrate)	-	0.8 g
10% Citric acid (monohydrate)	-	0.055 g
Sod. Chloride	-	0.42 g
Distilled water	-	100 ml

10 percent formal saline:

For 1 litre:

Formaline (40%)	-	100 ml
NaCl	-	8.5 g
Distilled water	-	900 ml

Feed composition of the experimental rats

One kg of feed contains:

Ground maize	-	673.2 g
Soyabean extract	-	282.3 g
Mineral mix.	-	044.4 g

Protocol of the haemagglutination (HA) test