CHAPTER II

Material and Methods

In present project it is proposed to study the toxicity of Nag bhasma bhasma in male albino rats. The albino rats and other material used throughout the project are described in this chapter along with the details of methods used to test toxicity of nag bhasma, The histological methods and bioassay methods used to study different parameters are given in detail.

ANIMALS

Male albino rats that were originally derived from Haffkine strain and were bred and maintained in departmental animal house. The rats were fed standard pellet diet rat feed prepared by Amrit feeds (Navmaharashtra Chakan Oil Mills, Sangli, Maharashtra, India) and water *ad libitum* during breeding, maintenance and experimental schedule. The male albino rats 90 days old, weighing 100-110 g were used for experimental work.

CHEMICALS

p-nitrophenyl phosphate, *p*-nitrophenyl-β-D-glucosiduronic acid, bovine haemoglobin, Bovine serum albumin, RNA, DNA, orange G, Hematoxylin, aniline blue, ditthiozone were purchased from Sigma Chemical Company, St. Louis, USA. All other chemicals were of the highest purity obtained from BDH and E. Merk, India. Pathological diagnostic kits were purchased from Nirmal laboratory, Chopada (Jalagaon), India.

Doses of lead nitrate (20 mg/kg body wt) and Nag bhasma (30 mg/kg, 60 mg/kg and 90 mg/kg body wt.) were given daily to albino rats for 7, 14 and 21 days. Simultaneously control rats were maintained. In these rats various parameters were studied to evaluate the toxicity.

1. Nagbhasma preparation (Ayurveda Sarsangrah, 1971):

Nag bhasma was prepared from lead through the processes of shodhan and maran as described below-

Shodhan :

This procedure has been described in Ayurveda and Rasa Shastra for the elimination of toxic effects of lead. It is done as given below-

Take good quality lead from authenticated metallurgist. Melt it in a pan and quench in sesame oil. Repeat the procedure for 7 times. Similarly melt lead and quench sequentially for seven times in buttermilk, urine of cow, kanji prepared by the fermentation of rice and decoction of horse-gram. The lead becomes pure as far as the Ayurvedic pharmacology is concerned.

Maran (Bhasmikaran) :

Melt lead in an iron pan. Then add to it the fine powder of the bark of *ficus religiosa* slowly with constant stirring till red coloured fine amorphous powder is formed. Then close the pan with lid and heat strongly till red-hot. Cool it and strain it through fine meshed cotton cloth. Mix with equal quantity of manahsheela, triturate with the leaf juice of *Adathoda vasica* then prepare pellets of large size and incinerate with fire of 10 to 12 pieces of wood. Repeat this incinerates in process 10 times to form nirutha Nag bhasma, but reduce the quantity of manahsheela.

Test of quality :

The quality of Nag bhasma was tested as illustrated in Ayurveda and Rasa Shastra by heating the known quantity of Nag bhasma mixed

with the equal quantity of mitra panchaka { ghee, guggulu, gunja (seeds of Abrus), jauggary and borax } incinerate strongly on a fire.

The Nag bhasma prepared for present study was pure as no metal particles were appeared after incinerating Nag bhasma with mitra panchaka.

2. Preparation of Nag bhasma suspension :

The suspension was prepared by suspending 20 mg of Nag bhasma/ml of 25 % sugar solution. A required dose was given to each of the rats in different groups.

Similarly suspension of lead nitrate was also prepared for control groups of rats. A required dose was given to each of the rats in different groups.

3.Experimental protocol :

The rats were given the treatments as described below.

The animals of specification described above were divided into main five groups each containing 7 animals.

Group I : Normal rats - Normal rats (21) with the above description were maintained as normal Every time 7 animals were sacrificed on 8th , 15th and 21st day of the treatments to the rats of the groups 2 to 5 .

Group II: $Pb(NO_3)_2$ treated control rats – 20 mg/kg body wt. $Pb(NO_3)_2$ was fed in sugar suspention to the rats daily between 8.00 – 9.00 a.m. and 7 rats were sacrificed on 8th day. Rest of the rats further received the treatment of Nag bhasma. Again 7 rats were killed on 15th day after the treatment of Nag bhasma for 14 days. The treatments of lead nitrate to remaining 7 rats was continued further until 21 days and were killed on 22nd day.

Group III : 30 mg/kg body wt Nag bhasma treated rats - The animals (21) were given orally 30 mg/kg body wt. Nag bhasma in a sugar suspension to the rats daily between 8.00 – 9.00 a. m. and 7 rats were sacrificed on 8th day after giving 7 doses of Nag bhasma. The treatment of Nag bhasma to remaining 14 rats was continued further and 7 rats were killed on 15th day after receiving Nag bhasma for 14 days. Rest of the rats (7) were given further treatment of Nag bhasma until 21 days and killed on 22nd day.

Group IV : 60 mg/kg body wt Nag bhasma treated rats – Twenty rats of were given orally 60 mg/kg body wt. Nag bhasma as sugar suspesion

daily between 8.00 –9.00 a. m. Seven rats were killed on 8th day after the animals received 7doses. Rest of the rats was further given Nag bhasma treatment. Seven rats were killed on 15th day after the treatment of 14 days. Remaining 7 rats were given Nag bhasma treatment continuously until 21st day and were killed on 22nd day.

Group V : 90 mg/kg body wt Nag bhasma treated rats - Twenty one animals received daily 90 mg/kg body wt Nag bhasma in sugar suspension (po) 8.00 - 9.00 a.m. for 7 days and seven rats were sacrificed on 8th day after 7 doses. The treatment to the rest of the rats was continued later. Seven rats were killed on 15th day after receiving Nag Bhasma for 14 days. Remaining 7 rats were further given the treatment of Nag bhasma until 21st day and were killed on 22nd day.

Parameters Studied:

A. Serological parameters

Collection of serum - On the completion of the experimental schedule the animals were killed by giving deep ether anaesthesia. The blood samples were aspirated from the left ventricle with the disposable syringes and were allowed to clot at room temperature. On clotting the serum samples were obtained by centrifuging the clots using tabletop centrifuge. The colourless samples were stored at 10° C until use.

Liver Function Tests :

Diagnosis of liver function in human is performed using serum levels of AST/Aspartate aminotransferase/GOT, ALT/alanine aminotransferase/GPT, Alkaline phosphatase (ALP) and bilirubin (conjugated and nonconjugated). Alkaline phosphatase (ALP) and bilirubin (conjugated and nonconjugated) did not show significant alterations during present study and the data is not given. Therefore in the present study liver function tests too were studied in albino rats.

AST, ALT and bilirubin were estimated using commercial kits available from Nirmal laboratory, Chopada, Jalagaon). The assay methods of AST and ALT were as described by Reitman and Frankel [1957] and that of bilirubin was as described by Jendlrassik and Groï (1938). Alkaline phosphatase was assayed according to the method described by Bergmeyer [1965].

Assay of alkaline phosphatase:

Chemicals-

1. Subhstrate buffer -

Glycine	375 mg
NaOH	166 mg

$MgCl_2$	10 mg
p-nitrophenyl phosphate	165 mg

Dissolve above chemicals in distilled water and make final volume 100 ml. The pH of the solution will be 9.2

2. NaOH (0.02 N)- Dissolve 80 mg NaOH in 100 ml distilled water.

Assay of serum alkaline phosphatese:

Take 1.0 ml substrate buffer and 0.1 ml suitably diluted serum and incubate at 37° C for 30 minutes. Stop the reaction by the addition of 10 ml 0.02 N NaOH. Do not add the serum to control tube during incubation. Add serum to control tube stopping the enzyme activity by NaOH. Take reading at 405 nm. Calculate the activity by converting OD to the amount of p-nitrophenol released.

Unit of enzyme activity was defined as the amount of *p*-nitrophenyl phosphate required releasing 1μM of *p*-nitrophenol per minute under standard assay conditions.

A. Histology:

Normal histological appearance of any organ reflects normal physiology of any animal. Histological alterations are used for the

diagnosis of pathological condition. During toxicological and pathological studies the variations in the histology are exploited for the evaluation of physiological state of the animal. Therefore during present work also the histopathological alterations were studied.

Preparation of tissue for histology:

Always pieces were selected from central region of central (main lobe of liver for histological studies. Kidneys were removed and were stripped off of peritoneal wall and fat. The middle region pieces were used for studies ensuring that circular piece/half circular piece is selected. Always new razor blade was used to cut tissue into pieces.

Fixation and microtechnique :

Livers and kidneys were dissected out and cut with the precautions described above into pieces of 0.5 x 0.5 x 0.5 cm, fixed in Bouin's fixative, 10% buffered paraformaldehyde. The fixatives were prepared as follows

Bouin's fixative :

It was prepared as described by Thompson (1966).

1. Saturated picric acid (trinitrophenol) 20 g

   Distilled water 1000 ml

   Dissolve picric acid in distilled water with the aid of heat, allow to cool and decant the supernatant

2. Bouin's stalk solution

   Solution 1 750 ml

   Paraformaldehyde (4%) 250 ml

3. Bouin's fixative

   Solution 2 95 ml

   Picric acid 5 ml

   Add glacial acetic aid to stock Bouin's fluid just before the fixative is to be used. The pH of this fixative should be approximately 1.5 to 1.7.

1) Buffered 10 % formalin :

a) Phosphate buffer

Dibasic anhydrous sodium phosphate (Na_2HPO_4) 6.5 g

Monobasic acid potassium phosphate (KH_2PO_4) 4.0 g

B] Paraformaldehyde

Dissolve 80 -g paraformaldehyde in distilled water by heating at 60° C till complete dissolution. Mix equal volumes of phosphate buffer and paraformaldehyde solution. pH of this fixative should be 7.00

The tissues were processed for wax sectioning as describe by Pears (1968) and Thompson (1966). The sections were cut of 0.5 µ a stained with hematoxylin and eosin and with Mallory's triple stain.

Preparation of chemicals:

1. Harris' haematoxylin

Haematoxylin (national aniline No. 564)	5.0 g
Ethyl alcohol (absolute)	50.0 ml
Aluminum ammonium sulfate	100.0 g
Mercuric oxide	2.5 g
Distilled water	1000.0 ml

Dissolve aluminum ammonium sulfate in 900 ml boiling water. Similarly dissolve 5 g haematoxylin in small quantity of absolute alcohol. Mix above two solutions and boil rapidly. Remove from the heat and add mercuric

oxide very slowly. Shake well and plunge in a cold water. This is ready for use as soon as it cools and this stain can be stored indefinitely.

2. Eosin

Eosin Y (National aniline No. 516)	2.0 g
Absolute ethyl alcohol (AA)	200.0 ml

Dissolve eosin in alcohol and filter the stain.

3. Mallory's aniline blue

i. 1 % alcoholic iodine

Iodine	1.0 g
AA	100.0 ml

ii. 5 % sodium thiosulfate

Sodium thiosulfate	5.0 g
Distilled water (Dist. W)	100.0 ml

iii. 0.5 % aqueous acid fuschin (national aniline No. 402)

Acid fuschin	0.5 mg
Distilled water	100.0 ml

iv. Aniline blue – orange G

Aniline blue	0.5 g
(Water soluble national aniline No.426)	
Orange G 9 national aniline No. 696)	2.0 g
Phosphoric acid	1.0 g
Distilled water	100.0 ml

Mix all the above chemicals and use.

Staining procedures:

A] Hematoxylin and eosin

1) Dewax the sections for 30' in xylene

2) Hydrate through descending alcohol grades

3) Wash with distilled water

4) Treat the sections with Harris' Hematoxylin for 8 minute at room temperature

5) Wash with distilled water repeatedly

6) Dehydrate through ascending grade of alcohol

7) Bring slides in absolute alcohol

8) Stain with eosin for 1 minute

9) Wash with absolute alcohol

10) clear in xylene

11) Mount in DPX

To ensure the nuclear staining two other methods were used.

A1-Toludine Blue O pH 3.oo

Chemicals—

i) 0.1N HCl

ii) Deoxyribonuclease solution in acetate buffer 0.1 M pH 4.8

iii) Ribonuclease solution in acetate buffer 0.1 M pH 4.8

iv) 0.5% Toludine Blue O pH 3.00---In aqueous solution prepare solution and adjust the pH with solution I

v) 0.5% Methylene Blue pH 3.00---In aqueous solution prepare solution and adjust the pH with solution I

vi) 5% ammonium molybdate aqueous. (use it in case of Ribonuclease treatment.)

Procedure:--

1.Use wax slides fixed in 10% formaldehyde

2.Bring slides to distilled water through descending alcohol grades.

3.Prior the nuclear staining either by, 0.5% Toludine Blue O pH3.00 or by 0.5% Methylene Blue pH 3.00 digest the DNA in one slide and RNA in other slide in buffered solutions of Deoxyribonuclease (chemical ii) and Ribonuclease (chemical iii) respectively and confirm their presence and hence nuclear staining in slides directly stained with 0.5% Toludine Blue pH3.00 0.5 % Methylene Blue pH 3.00.

For the microphotographs to depict the histology the sections were stained with toludine blue and eosin.

B - Mallory's aniline blue stain

a) Dewax the slides in xylene for 30'

b) Bring the slides to absolute alcohol

c) Treat the section with 1% alcoholic iodine for 5'

d) Wash with water

e) Treat the slides with 5% acid fuschin for 5'

f) Treat the sections with aniline blue – orange G for 50 to 60'

g) Wash with 95 % alcohol

h) Wash with absolute alcohol

i) Clear in xylene and mount in DPX.

C) Histochemical demonstration of haemoglobin in RBCs

Haemoglobin was demonstrated histochemically as described by Thompson (1966).

Chemicals:

1% benzidine in methanol

20% hydrogen peroxide in ethyl alcohol

Procedure :

1 Prepare the blood thin smears and air dry.

2 Treat with hydrogen peroxide for 1 second.

3 Pour off the solution and treat with beizidine

4 Pour off and wash the smear with distilled water

5 Mount the smears in 50% glycerol or PVP (10 polyvinyl pyrollidon in 10 ml distilled water)

D) Histochemical demonstration of lead by Sodium Rhodizonate (Feigl, 1966).

1.Chemicals:

i) 0.2% Neutral Sodium Rhodizonate ($Na_2C_6O_6$) 0.2 g

Sodium Rhodizonate($Na_2C_6O_6$) 0.2 g

Distilled Water 100.0 ml.

ii) Sodium Rhodizonate pH 3.0 :- Take 50ml of solution i) gradually add drop by drop glacial acetic acid until pH 3.00 is attained.

iii) 20% HCl-20ml HCl + 80 ml distilled water.

Procedure

1.Use wax sections fixed in 10% buffered formaldehyde.

2.Treat one slide with solution i and other with solution ii for 60'

3.Rinse with distilled water.

4.Treat with 20% HCl 5'

5.Wash with distilled water 5'

6.Dehydrate through ascending alcohol grades.

7. Clear in xylene.

8. Mount in DPX.

**Proper control slides were carried out to confirm the lead reaction.

The distribution of lead was further confirmed by Mallory's phloxin stain which is also used to demonstrate lead depositing bodies (Thompson, 1966).

Chemicals

I. Harris' Hematoxylin

II. 1% acid alcohol

III. Ammonia water

IV. 0.5% Mallory's alcoholic phloxine B

Absolute ethyl alcohol	1g
Distilled water to make	200 ml.

V. 0.1% Aqueous lithium carbonate

Lithium carbonate	0.1 g
Distilled water	100 ml

Procedure

1. Bring slides to distilled water

2. Treat with solution I for 15'

3. Wash with distilled water for 2'

4. Differentiate in solution No. II

5. Wash with distilled water for 2'

6. Give a dip in ammonia water.

7. Wash with distilled water for 2'

8.Treat with solution V for 2'

9.Wash with distilled water for 2'

10.Make a permanent preparation.

The microphotographs that depict the histology are prepared from the TB - pH 3.00 and counter stained with eosin.

B. BIOCHEMICAL ASSAYS:

Preparations of tissue homogenates:

The pieces of liver from central region of central lobe, full transverse sections of kidney were used ensuring the inclusion of medulla and cortex in natural proportion and Known quantities of liver, kidney were homogenised in Teflon homogenizer with 4 strokes at 1300 rpm. The homogenates were diluted with distilled water to a known volume.

Biochemical assays of lysosomal enzymes :

Three lysosomal enzymes, *viz.* Acid phosphatase, β-glucuronidase, cathepsin D, were studied biochemicaly. Following assay methods were used for the estimation of the above enzymes-

Acid phosphatase :

Acid phosphaatase activity was determined according to Linhardt and Walter (1965)

Preparation of chemicals –

I. Citrate buffer (0.1 M, pH 4.8)

Dissolve 410 mg citric acid and 1.125 g sodium citrate in distilled water and make final volume of 100 ml.

II. Substrate buffer solution

Dissolve 165 mg ρ - nitrophenyl phosphate in 100 ml above citrate buffer.

III. NaOH (0.1 N)

Dissolve 4.0 g NaOH in a final volume of 1000 ml

Assay of acid phosphatase

Solution	Blank ml	Sample ml
Substrate buffer	1.0	1.0
Distilled water	0.2	0.0
Tissue homogenate	0.0	0.2

Incubate the tubes at 37° for 30 minutes. The enzyme reaction was arrested by adding 4 ml NaOH (0.1N). Released ρ - nitrophenol becomes yellow in colour by the addition of NaOH. The readings were taken at 405 μM on spectronic 20 (Bosch and Lomb). The optical density was converted into the concentration of ρ - nitrophenol using the standard graph of ρ - nitrophenol against optical density.

Enzyme unit – one unit of acid phosphatase was defined as the amount of enzyme required to release 1 μM of ρ - nitrophenol/min from ρ-nitrophenyl phosphate under standard assay conditions.

β-Glucuronidase :

Biochemical assay of β-glucuronidase was carried out according to Himeno *et al* (1972)

Preparations of chemicals-

I. acetate buffer (0.1 M, pH 4.4)

Dissolve 579-mg sodium acetate in distilled water in50 ml distilled water and adjust pH to 4.4 with 0.325-ml glacial acetic acid and dilute to a final volume of 100 ml.

II. Substrate buffer

Dissolve 5.0-mg ρ-nitrophenyl-β-D-glucosiduronic acid in 100 ml of above buffer.

III. NaOH (0.1 N)

Dissolve 4.0 g NaOH in a final volume of 1000 ml

Assay of β-glucuronidase

Solution	Blank ml	Sample ml
Substrate buffer	1.0	1.0
Distilled water	0.2	0.0
Tissue homogenate	0.0	0.2

Incubate the tubes for 30 minutes at 37°. The enzyme activity was stopped by adding 4 ml NaOH (0.1 N). Released ρ-nitrophenol becomes yellow at alkaline pH due by the addition of NaOH. The readings were taken at 405 μM on spectronic 20 (Bosch and Lomb). The optical density was converted into the concentration of ρ-nitrophenol using the standard graph of ρ - nitrophenol against optical density.

Enzyme unit – one unit of β-glucuronidase was defined as the amount of enzyme required to release 1 μM of ρ-nitrophenol/min from ρ-nitrophenyl-β-D-glucosiduronic acid under standard assay conditions.

Cathepsin D activity:

It was determined by the method of Mycek (1970).

Preparation of chemicals-

I. Bovine haemoglobin solution at pH 3.0

Dissolve bovine haemoglobin in few ml of distilled water and adjust pH to 1.8 with 1 N HCl to denature it and wait for 15 minutes. The raise the pH to 3.0 by the addition of 1 N NaOH. Make final volume to 100 ml and filter the solution with Whatman No. 1 filter paper.

II. TCA (10)

Dissolve 10-g trichloroacetic acid in 100 ml distilled water.

Assay of Cathepsin D

Solution	Blank ml	Sample ml
Substrate buffer	1.0	1.0
Tissue homogenate	0.0	0.25

The above mixture was incubated for 30 minutes at 37° C. The reaction was Stopped by adding 2 ml 10 % TCA. Similarly 0.25 ml homogenate was added to the blank tubes. The tubes Centrifuged at 1000 x g for 10 minutes and the supernatant was poured into the clean dry test tube. Readings were taken at 280 nM on UV-Visible spectrophotometer using blank as a reference

Enzyme unit- one unit of enzyme is the amount of enzyme required for the release of amino acids from bovine haemoglobin to change the optical density by 1.0.

Estimation of total proteins :

Total proteins were estimated using Folin-Ciocalteu phenol reagent as described by Lowry *et al* (1951).

Preparation of chemicals-

I. Lowry's A –

2 % sodium carbonate in 0.1 N NaOH.

II. Lowry's B –

0.5 % copper sulphate in 1 % sodium tartarate at pH 7.0.

III. Lowry's C –

50 ml Lowry's A + 1 ml Lowry's B. Prepare this reagent fresh just prior to use.

IV. Folin-Ciocalteu phenol reagent-

Sodium tungstate [$Na_2WO_4.2H_2O$]	100 g
Sodium molybdate [$Na_2MoO_4.2H_2O$]	25 g
Distilled water	700 ml
Conc. HCl	100 ml
Phosphoric acid	50 ml

Reflux the above mixture for 10 hr in glass apparatus

Then add to this mixture

Lithium sulphate	150 g
Distilled water	50 ml

Add five drops of bromine water and boil this to remove excess bromine. Cool and dilute to 1.0 N.

Assay of proteins

Solution	Blank ml	Sample ml
H_2O	1.5	1.3
Homogenate	0.0	0.2
Lowry's C	3.0	3.0
	Wait for 15 minutes	
Folin-Ciocalteu reagent	0.5	0.5

Wait for 60 minute and take readings at 660 nm.

Convert optical density to protein value from the standard graph plotted as optical density verses concentration of standard albumin. Calculate the amount of protein per g tissue.

Statistical analysis

The results of various experiments were analysed statistically. The statistical calculations were carried out according to the method given in the textbook of statistics (Agarwaal, 1990). The following abbreviations and formulae were used:

X – independent variable

N – number of observations

Formulae –

1. $\bar{x}$ = arithmatic mean of x

2. $$\bar{x} = \frac{x}{n}$$

SD – standard deviation

$$SD = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{n - 2}}$$

$$SE = \frac{SD}{n}$$

SE – Standard error for a mean of n variables.

Students t – test

$$t = \frac{\bar{x}_1 - \bar{x}_2}{E_1 - E_2}$$

Where $\bar{x}_1 - \bar{x}_2$ is mean difference of two variables and

$(SE_1 - SE_2)$ = Error of the mean difference

$$= \sqrt{\frac{SD_1^2}{n_1} + \frac{SD_1^2}{n_2}}$$

df = Degree of freedom for a differences of two means of n_1 and n_2 variables.

$$df = n_1 + n_2 - 2$$

Significance – The probability *p* of obtained 't' value at least as calculated for a given number of degree of freedom is given in Fischer's table (Fischer, 1938). The *p* values are signified according to the following conventions-

$P > 0.05$ the difference is said to be insignificant

$P < 0.05$ the difference is said to be almost significant

$P < 0.02$ the difference is said to be significant

$P < 0.001$ the difference is said to be highly significant