

Material and methods experimental design biology essay

[Science](#), [Biology](#)



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The current study involves the investigation of hematological and serological alterations, electrophoretic analysis of the serum protein profile and the histopathological changes in the liver tissues of rats treated with high doses of iron and copper via intraperitoneal administration against a control group. To investigate serological changes, different parameters of liver function test (LFT), lipid profile and renal function test (RFT) were studied using commercially available different kits. The blood profile was carried out on the automated hematology analyzer (Model MEK-6318 K, Power Input 190 VA, 220-240V, Nihon Kohen Corp). Study of protein profile was carried out by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) which resolves the protein fractions on the basis of their molecular weight. Histopathological changes were observed by using Hematoxylin & Eosin, Prussian blue and Rhodanine staining.

Animals and doses:

The colonies of Wistar Rats were obtained and reared at the Department of Zoology, University of the Punjab, Lahore. They were kept in a fully aerated room at room temperature 24-25°C and were provided with the normal Rat Chow (20% crude proteins) and water ad libitum. Weight of all the animals was monitored twice a week and the experimental work was performed when the Rats reached a weight of 200 ± 25 g. Three experimental groups (n= 9) were established against a control group (n= 3).

Figure: Dose Plan

Preparation of Doses:

For dose preparation, hydrous ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and cupric sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) were dissolved in distilled water separately and mutually as well, half an hour prior to the experiment. The doses were administered intra-peritoneally to the rats. The animals were tagged properly and were allowed for normal activities. They were under constant observation after the dose administration to study their behavior. The animals were sacrificed 24h after the intraperitoneal administration of the dose.

Dissections

The experimental rats were anesthetized by I. P. (Intraperitoneal) administration of Ketamine distilled water mixture (1: 1). After the animals were anesthetized the fur was saturated with ethanol and a small incision was made to cut the abdominal wall with sharp scissors. Then the muscular layer was cut on the sides to expose the internal organs. 0. 9% saline solution was poured on the exposed organs of animal in order to avoid drying. The dissections were done in aseptic maintained conditions to draw the blood through direct cardiac puncture and excise the liver out.

Blood Sampling and Processing

The blood samples were collected in sterilized disposable syringes (Becton Dickinson, Private Ltd.), 2ml of the blood was transferred to K3-EDTA coated vacutainers (Becton Dickinson, Private Ltd.) for complete blood count and, 6ml in vacutainers (without any clotting factor) to separate the serum. For

serum separation, the blood was kept for 2-3 hours at room temperature and then centrifuged at 4000 rpm for 20 minutes. The serum was collected in new labeled eppendorf cups and was stored at -20°C, till further use.

Tissue Sampling and Processing

Liver of each animal was obtained after dissection and was placed in Petri dish containing 0.9% saline, cut into 1×1cm pieces and stored with 10% formalin in labeled glass bottles until further processed. Tissues for the proteomic analysis were frozen at -20°C immediately after dissection.

Methods in Clinical Chemistry:

Complete Blood Count Analysis:

Blood in EDTA containing vacutainers was kept on a shaker. Blood indices were analyzed using automated hematology analyzer (Model MEK-6318; Power input 190V A; 220-240 Volts; Nihon Kohden Corp.).

Serum Analysis:

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

(SDS-PAGE)

Reagent Preparation

Acrylamide-Bisacrylamide (30%)

To prepare 30% solution of acrylamide-bisacrylamide 58.40g of acrylamide and 1.6g of bis-acrylamide were dissolved in 100 ml of distilled water using magnetic stirrer. Once the clear solution was obtained the total volume was

made up to 100ml with distilled water using volumetric cylinder and stored in at 4°C in a refrigerator.

Tris-HCl (pH 8.8, 3.0 M)

To prepare a 3M solution of Tris-HCl, 36.3g of Trizma base [Tris (Hydroxymethyl) amino methane] was dissolved in distilled water using magnetic stirrer. The pH of the solution was adjusted to 8.8 using concentrated HCl. Finally the volume was made volume up to 100ml with distilled water using a volumetric cylinder and stored at 4°C in a refrigerator.

Tris-HCl (pH 6.8, 1.0 M)

1M Tris-HCl was prepared by dissolving the 12.11g Trizma base [Tris (Hydroxymethyl) amino methane] in 100 ml of distilled water (final volume) with the help of a magnetic stirrer. The pH of the solution was adjusted to 6.8 using concentrated HCl and stored at 4°C.

SDS 10%

10% of the SDS was prepared by dissolving 10g of SDS in 80ml of distilled water on a magnetic stirrer. Once the SDS was dissolved completely the final volume was made up to 100 ml and the solution was stored at room temperature.

Electrophoresis Buffer (running buffer)

To prepare 1X of the running buffer 3g of Tris, 14.4g of glycine and 1g of SDS were dissolved in distilled water in a beaker. Final volume of the solution was made up to 1 liter with distilled water in a volumetric cylinder and stored at 4°C.

Loading Dye

154mg of Dithiothreitol (DTT) and 200mg of SDS was dissolved in 8ml of 1M Tris (pH 6.8). 10ml of glycerol was added to the mixture and when the glycerol was dissolved 20mg of bromophenol blue dye was added in the solution and mixed completely. The loading dye was aliquot in 1.5ml eppendorf tubes covered with aluminum foils and stored at 4°C.

Staining Solution

125 mg of Coomassie Blue R250 was taken in a stopper flask and 112.5ml methanol, 22.5ml of acetic acid and 112.5ml of distilled water was added in the flask. The solution was dissolved completely using magnetic stirrer and stored in a dark bottle at room temperature.

Destaining Solution

50ml of methanol and 70ml of acetic acid were mixed and the final volume was made up to 1 liter in a graduated cylinder using distilled water and stored at room temperature.

Preparation of Working Dilutions

Serum samples were diluted with loading dye and electrophoresis Buffer to prepare the working dilutions for gel electrophoresis. For this purpose, serum samples, each containing 18µg of protein concentration was added in electrophoresis buffer to make total volume of 6µl in new eppendorf tubes. The samples were gently but thoroughly mixed using vortex and heated in a boiling water bath for 5 minutes to denature the proteins. Then 6µl of loading

dye was added in each eppendorf tube. After that, 10µl of each sample was loaded on to the gel.

Protein Markers

10 µl of Fermentas PageRuler™ unstained protein ladder # SM0661 was loaded in first well. There were 14 different reference bands in the marker.

METHODS IN HISTOCHEMISTRY

Figure: Flow sheet depicting the steps in Histology

Tissue Processing:

Fixation

Liver was excised, cut into 1×1cm pieces and the pieces were immediately preserved by placing them in 10% formalin solution. Fixation is required to prevent autolysis and microbial degradation.

Dehydration

Fixed tissue samples were rinsed in tap water and were then allowed to dehydrate by passing through ascending grades of ethanol solution. 40% Ethanol over night 50% Ethanol over night 70% Ethanol 72 hours 90% Ethanol over night 100% Ethanol 6 hours

Clearing:

Once the dehydration was done, tissues were placed in xylene at 25°C with two changes for 12 hours each. As xylene is miscible with both alcohol and paraffin, it was used as a clearing agent to remove alcohol from the tissues.

Infiltration:

After clearing, tissues were incubated in 100% paraffin wax at 58°C-60°C over night. At high temperature, xylene was evaporated and the tissue spaces were infiltrated with paraffin.

Figure: Flow sheet showing steps in Tissue Processing**Embedding:**

Molten wax was poured in cavity blocks, already greased with clove oil. As the wax began to solidify in the base, the tissue was placed in it using the forceps with proper orientation in order to represent appropriate morphology. Molten wax was poured in it to fill up the cavity and then air bubbles were removed with the help of hot needle. It was then allowed to solidify.

Mounting:

Paraffin block along with embedded tissue was then trimmed to remove extra wax and a square block was made. It was then fixed on a wooden block with the help of melted wax and allowed to dry.

Microtomy (Sectioning):

The paraffin blocks were inserted into the microtome chuck. Fresh blade was used and the blade holder was positioned in a way so that the wax block and the blade lie almost parallel to each other. As the sectioning started, first few ribbons were discarded until a ribbon with tissue section was obtained. To avoid wrinkling, ribbons were carefully handled with a camel hair brush. When the ribbon of desired length was obtained, it was separated from the

blade and was placed on a clear surface. It was then picked up with a forceps and a camel hair brush and was floated on the surface of water bath, already set at a temperature of 40-45°C. As the ribbon became straight, it was mounted on the clean glass slide, coated with Mayer's adhesive (Albumin in Mayer's adhesive helps to stick the ribbon on slide). Afterwards, slides were incubated in an oven at 45°C to dry and affix the ribbon on them. Slides were then stored at 4°C until further use.

Preparation of Slides:

Preparation of Mayer's Adhesive:

Egg White 50%Glycerol 50%Thymol (fungicide) few dropsSlides were coated with Mayer's Adhesive with the help of fingertip, as it is helpful to form a very thin and even layer on the slide. These slides were placed in an incubator for drying and were wrapped with tissue paper and were stored at 4°C for further use.

STAINING:

H & E Staining:

Preparation of stains:

Harris Hematoxylin:

Hematoxylin crystals 2. 5gEthyl Alcohol 25mlPotassium alum 50gDistilled water 250mlMercuric oxide 1. 25gHematoxylin was dissolved in the alcohol and potassium alum was dissolved in water by heating the solutions. Both solutions were mixed together and the mixture was boiled. Then mercuric acid was added slowly in the mixture and it was reheated until it turned into

dark purple. After that, the vessel containing mixture was immersed in cold water. (Addition of 4ml of glacial acetic acid per 100ml of solution increases the precision of the nuclear stain). The prepared stain was then filtered.

Eosin Y Working Solution (0.5%):

Eosin Y 0.5g 70% Ethanol 100ml Eosin was added in 70% ethanol, mixed well and then stored at room temperature.

H & E Staining Protocol:

The slides containing sections were deparaffinized in two changes of xylene, 30 minutes each at 50°C. Rehydration was carried out by passing the slides through a series of descending grades of alcohol: Absolute Ethanol for 5 minutes 95% Ethanol for 5 minutes 70% Ethanol for 5 minutes 50% Ethanol for 3 minutes 30% Ethanol for 3 minutes After that, slides were washed in distilled water for 5 minutes. Sections mounted on slides were then stained with Harris hematoxylin solution for 1.5 minutes. Slides were dipped in acidic alcohol for 1 second in order to remove excess Harris hematoxylin. Slides were then washed in distilled water for 15 minutes, followed by dehydration with 70% ethanol for 5 minutes and 90% ethanol for 5 minutes. Counter staining was done by immersing the slides in eosin solution for 3-5 minutes, and it was followed by absolute ethanol for 5 minutes. The sections were then cleared with xylene for 5 minutes. Finally the slides were mounted with coverslips using Canada balsam.

Iron Staining:

Solutions And Reagents Required:

20% Aqueous Solution of Hydrochloric Acid:

Hydrochloric acid, concentrated 20 ml
Distilled water 80 ml

10% Aqueous Solution of Potassium Ferrocyanide:

Potassium ferrocyanide, trihydrate 10 g
Distilled water 100 ml
Potassium ferrocyanide was dissolved in distilled water by continuous stirring.

Nuclear Fast Red Solution:

Nuclear fast red 0.1 g
Aluminum sulfate 5 g
Distilled water 100 ml
Aluminum sulfate was dissolved in water. Then nuclear fast red was added in the solution and heated to boil. After cooling, the solution was filtered and a grain of thymol was added as a preservative.

Working Solution:

Equal parts of 20% hydrochloric acid and 10% potassium ferrocyanide solutions were mixed together just before use.

Staining Procedure:

The slides containing sections were deparaffinized in two changes of xylene, 30 minutes each at 50°C. Rehydration was carried out by passing the slides through a series of descending grades of alcohol: Absolute Ethanol for 5 minutes
95% Ethanol for 5 minutes
70% Ethanol for 5 minutes
50% Ethanol for 3 minutes
30% Ethanol for 3 minutes
After that, slides were washed in distilled water for 5 minutes. Equal parts of 20% hydrochloric acid and 10%

potassium ferrocyanide were mixed for working solution. Slides were immersed in this solution for 20 minutes. These slides were rinsed in several changes of distilled water. Sections mounted on the slides were stained in nuclear fast red for 2 to 5 minutes, depending on the intensity of the counterstain required. Washing was done with tap water. Sections were dehydrated through ascending grades of alcohol, with 70% ethanol for 5 minutes and 90% ethanol for 5 minutes; it was followed by absolute ethanol for 5 minutes and were cleared in xylene with 2 changes. Finally the slides were mounted with coverslips using Canada balsam.

Rhodanine Staining:

Solutions and Reagents Required:

Rhodanine Stock Solution:

5-p-dimethylaminobenzylidene rhodanine 0.2 gm
Absolute alcohol 100.0 ml
Solution was mixed well and refrigerated. Solution can be stable for 3 months.

Rhodanine Working Solution:

Stock Rhodanine 10.0 ml
Distilled water 40.0 ml
Stock solution was shaken before measuring and was filtered through coarse filter paper. Solution was freshly prepared and discarded after use.

Hematoxylin:

Commercial Gill-3

Hematoxylin 6g
Sodium iodate 0.6g
Aluminium sulphate 80g
Glacial acetic acid 20ml
Ethylene glycol 250ml
Distilled water 750ml
Ethylene glycol was

dissolved in distilled water, followed by addition of hematoxylin, then sodium iodate, aluminium sulphate and finally acetic acid. The whole mixture was stirred for 1 hour at room temperature.

Staining Procedure:

The slides containing sections were deparaffinized in two changes of xylene, 30 minutes each at 50°C. Rehydration was carried out by passing the slides through a series of descending grades of alcohol: Absolute Ethanol for 5 minutes, 95% Ethanol for 5 minutes, 70% Ethanol for 5 minutes, 50% Ethanol for 3 minutes, 30% Ethanol for 3 minutes. After that, slides were washed in distilled water for 5 minutes. Sections were dehydrated through ascending grades of alcohol, with 70% ethanol for 5 minutes and 90% ethanol for 5 minutes; it was then followed by absolute ethanol for 5 minutes and were cleared in xylene with 2 changes. Finally the slides were mounted with coverslips using Canada balsam.