



Effect of *Aspergillus spp.*, on Liver, Kidney and Intestines of WNIN Rat (*Rattus norvegicus*) Fed on Fungus Inoculated Rice (*Oryza sativa. L*) – An Electron Microscope Study

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Authors' contributions

This work was carried out by Principal author PMC designed and executed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Author VV provided the necessary lab inputs and suggestions where ever it was necessary. Author DRR is the Research Guide for author PMC Research work and managed the corrections of the first and second draft of the paper and taken part in the analyses and interpretations of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJNFS/2020/v12i1130324

Editor(s):

(1) Dr. Michael Lokuruka, Karatina University, Kenya.

(2) Dr. Adetunji Charles Oluwaseun, Edo University, Nigeria.

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Reviewers:

(1) B. R. Gurumurthy, Federal TVET Institute, Ethiopia.

(2) P. Ramanathan, Thanthai Hans Roever College, Autonomous, India.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/63021>

Original Research Article

Received 02 October 2020
Accepted 08 December 2020
Published 19 December 2020

ABSTRACT

Aims: This present study was conceived with two objectives. The first aim of the study is to establish the ultrastructural variation of normal rice grain collected from different market sources. Also, the study is aimed to investigate the fungal (*Aspergillus spp.*) infection in stored rice grain

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and Ultrastructural variation patterns in rice due to fungal infection by Scanning Electron Microscope (SEM). Further, the changes in selected visceral organs (Liver, Kidney and Intestines) of Wistar NIN Rat when they were fed on fungal inoculated rice.

Study Design: The study was completed in to the two phases.

First Phase: Collection of different rice samples, preparation of contaminated rice inoculated with *Aspergillus* sp., Ultrastructure analysis of rice samples by using SEM.

Second Phase: Animal experiments, Analysis of biochemical estimations in blood serum by spectrophotometer and ultrastructural studies in the selected visceral organs viz., Liver, kidney and intestines of rat by Transmission Electron Microscope (TEM) and Histopathological changes by Light microscope in rats fed with inoculated rice powder with *Aspergillus* spp.,(treated) fungus. Control rats were fed with normal rice powder (control).

Place and Duration of the Study: SEM Facility, Extension and Training division ICMR-National Institute of Nutrition, Tarnaka, Hyderabad. Telangana state, India. Duration from 2013 to 2015.

Methodology: Animal experiments with albino white Wistar Rat (*Rattus norvigecus*) weighing about approximately 120-140 grams of each rat and six (6) of each for treated group and control were taken for experimental purpose. Six rats were fed on inoculated rice with fungus *Aspergillus* sp., and 6 were fed without fungus inoculated rice for control. These animals were maintained under standard procedure as per the protocol of animal ethics. After 28 days of feeding, all the rats were sacrificed as per the regulations animal ethical guidelines. Tissues like liver, kidney and intestines of rats (treated and control) were processed and examined for histopathological and ultrastructural changes by using light and electron microscope. Blood serum from the treated and control rats were collected and processed for biochemical investigations by spectrophotometer.

Results: The hepatocytes in liver of the treatment group rats showed metachromatic granules (cytoplasmic) and nuclear pleomorphism (occurrence of more than one form of glycogen granules) existence in same species of more than one morphological type but it was absent in the control rats' livers. In electron microscope studies, swollen mitochondria and well developed smooth endoplasmic reticulum (SER) were present in treated group rats as against normal mitochondria and rough endoplasmic reticulum (RER) in control rats were observed. The kidneys of treatment group showed patch like mononuclear cell infiltrations in the cortex including many apoptotic bodies in between the renal tubules. Intestines of treated group rat showed the damages in the inner walls of intestinal epithelium in between cells. TEM studies showed swollen mitochondria, absorptive cells of epithelium and endoplasmic reticulum tubules were highly expanded in the treated group rat intestines while normal appearance of the intestines with well developed epithelial cells of microvilli was observed in control rats.

Conclusion: Improper storage of rice grains in food godowns would cause damage to the rice grains due to fungal infections. Although fungal infected grain apparently normal in appearance, from the food safety point of view the grains are not fit for human consumption. Hence, using SEM in quality control and assurance of food safety of rice grains to assess the quality to declare fitness for human consumption is required at this juncture.

Keywords: *Aspergillus* sp; liver; kidney; intestine; rat; light microscope (LM); Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM).

1. INTRODUCTION

Rice (*Oryza sativa*. L) is a staple food of the Southeast Asian population. Rice protein quality is of better as its protein efficiency ratio (PER) is superior as compared to other cereals and millets. Anatomically the kernel contains carbohydrates, proteins, micro and macro minerals and trace elements [1]. The structure and chemical composition of this cereal grain make it an ideal substrate for the establishment and growth of fungal species Factors like moisture content, water activity, temperature,

period of storage, initial levels of contamination, toxigenic potential of fungal strains and nature of the substrate influence the production of mycotoxins [2,3].

Aspergillus spp., is one of the most frequently found fungi cause severe damage either during growth of the grain in the plants before harvesting or during transport of grains to the godowns [4]. Also, many strains of the fungal infections produce significant doses of aflatoxins which cause illness in immune compromised individuals [5,6]. Thus, *Aspergillus* spp., produce

severe health and respiratory problems in human health [7,8]. Therefore, the present work was undertaken to study the cellular damage of different organs viz, Liver, Kidney and Intestines of treated rats. *Aspergillus sp.*, Infection is of a human health concern and animals globally. As there are no previous microscopic studies showing *Aspergillus sp.*, induced liver kidney and intestines damage and therefore, the present study was aimed to examine harmful effects of *Aspergillus sp.*, on rat tissues Liver, Kidney and Intestines using electron and light microscopy.

2. MATERIALS AND METHODS

2.1 Preparation of Growth of *Aspergillus spp.*, Fungus

Different samples of rice from different sources were collected from Hyderabad, Telangana state India. In each location 10 samples were taken from those rice bags stored for 12 to 24 months. Approximately 500 gms to 1kg rice grains collected carefully and packed in clean plastic bags and labeled. At least seven samples from different/varieties/cultures were collected and kept for study purpose. The first set of samples was left aside to inoculate *Aspergillus sp.*, while the second set of unsterilized samples were grounded to obtain powder and sterilized with sodium hypo chlorite (NaOCl) 1% for one minute and rinse 3-4 times in sterile water. These sterilized seeds were further processed as whole grain to obtain kernel powder. All these procedures were carried out under aseptic conditions and attempts were made to isolate the *Aspergillus spp.*, from the samples.

2.1.1 Cultures isolation of *Aspergillus spp.*, from the samples

Isolation of *Aspergillus spp.*, from first set of samples was done using agar plate method. As per the standard procedure about 400 seeds from each cultivar were placed on the agar plate 8 with replications for each cultivar and incubated at ambient temperature for 6 days. After six days of incubation period, each colony was identified with presence of fungus as per the standard procedure. The strain of the *Aspergillus sp.*, was isolated from rice kernels and cultured. The growth medium was prepared with rice and distilled water with quantity of 200gms rice and 120ml of distilled water in a 1L flask [9,10]. Cultures were incubated at room temperature (24°C) for 2 weeks and then transferred to an incubator at 10°C for further 2 weeks 50 ml of Vogel's minimal medium was inoculated

Saubouraud dextrose broth or potato dextrose broth to a final concentration of 10^7 spores / ml and incubated with shaking at 200 rpm until late exponential phase (18-24 h) at 37°C. The mycelium was isolated using Whatman 54 filter paper with a Buckner funnel and a side-arm flask attached to a vacuum pump and washed with 0.6 M MgSO₄. To freeze dry, the mycelium was transferred to a universal tube and refrigerated at - 80°C for a few hours. After few hours, the tube was sealed with parafilm and stored at room temperature Inoculated rice grains and control rice grains in the ratio of 1:1 was mixed dried and grounded to flour and used to feed the rats in experimental group [11,12].

2.2 Animal Experiment

A total number of twelve young adult albino white Wistar NIN (WNIN) male rats of 40 days of age, with an average body weight ranging from 125 to 140 gms each were used in this study from the colony of National Centre for Laboratory Animals Science (NCLAS). The experimental rats were randomly allocated into two groups of six each. Group I was control rats, fed with normal diet containing rice powder while Group II was experimental group which were fed with a ratio of 1:1 (vol/vol) mixture of *Aspergillus spp.*, inoculated rice diet for 28 days. Control rats were fed with normal rice diet for 28 days. Different doses of contaminated rice flour by weight containing *Aspergillus sp.*, 10^7 spores /ml were administered to the rats for 28 days. In order to avoid death of experimental animals due to starvation, the doses have been increased by weight based on the consumption level of the contaminated diet by the experimental rats during the experiment. However, the experimental animals were exposed at a constant strength of *Aspergillus spp.*, with of 10^7 spores /ml only. The animals were individually housed in stainless-steel cages, and maintained under standardized conditions of light with fresh tap water in polycarbonate bottles with stainless sipper tubes (Technoplast, Italy). They were housed at 22±2°C with 14-16 air changes per hour, with a relative humidity 50-60% and 12 hour light /dark cycle. After completion of 28 days, the 68 day's old rats were fasted for 24h and euthanized subjected to gross necropsy by using standard guidelines of ethical committee, blood was drawn for estimation of biochemical parameters like Hb serum Protein serum, Calcium, Phosphorous and enzyme like Alkaline Phosphatase (ALP) enzyme. External morphological features suggesting any

abnormality were further assessed by examining viscera *In situ* examination. Major organs liver kidneys and intestines were collected for Light Microscope (LM), SEM and TEM studies.

2.2.1 Tissue processing for light microscopy (LM) examinations

All tissues (liver, kidney and intestines) were washed with saline and used for Light microscopy studies by fixing in buffered 10% formalin (sodium phosphate buffer at pH 7.4) fixative solution. After fixation, specimens were subjected to routine histological processing such as embedding in paraffin wax [13] and then was cut in to 6 μm thick sections for frontal directions. The longitudinal sections (LS) and transverse sections (TS) were stained with Hematoxylin-Eosin (H&E), analyzed under a light microscope (Olympus BH-2 light microscope Olympus, Tokyo, Japan).

2.2.2 Sample preparation for Scanning Electron Microscope (SEM) studies

Tissues from liver, kidney and Intestines of control and treated rats were washed with normal saline and added cold Karnovsky's fixative (Paraformaldehyde, No. 989, Glutaraldehyde 25% batch no. 62930 Taab Laboratories Equipment Ltd., England UK, 0.2 M Sodium Cacodylate buffer (SCB) pH7.2 $\text{C}_2\text{H}_6\text{AsNaO}_2 \cdot 3\text{H}_2\text{O}$ Sigma USA C4945 MW 218.48 and Calcium Chloride RM170 Himedia Laboratories Pvt Ltd., Mumbai) to immerse the complete tissues in the fixative solution [14,15]. After one-hour, all above mentioned tissues were cut into slices 10-50mm H x W x L size by scalpel blade. Then a fresh fixative to all samples in cold overnight at 4°C for complete fixation was added.

First Washing: The samples were washed with working buffer (0.1M SCB) 3X times with 30 minutes interval to remove the excess fixative.

Post fixation: Buffered 2% Osmic acid (Osmium tetroxide OSO_4 CAS: 201816-12-0 *Loba Chemie Bombay* India) was added for 2 hours at 4°C.

Second washing: The samples were washed 3X times with 30mts intervals to remove excess post fixative with working buffer (0.1M) of Sodium cacodylate buffer.

Dehydration: All samples were dehydrated in ascending order of Ethanol (Ethyl alcohol 100%: Hayman Group Ltd., UK F204325) from 30,50,70,80,90-100% at 4°C for one hour. Then

100% ethanol was added and kept for another hour at room temperature for complete dehydration. All the samples were removed from the ethanol and air dried under high vacuum (10^{-7} Torr) at the room temperature (25°C) for one day followed by treatment of the samples with HMDS (1,1,1,3,3,3,Hexa Methyl Disilazane, *SRL Bombay no TT531534*) and dried under vacuum for 2 hours to complete drying [16]. All dried samples were mounted on aluminum stub with double sided adhesive tape (SPI supplies division of Structure Probe INC, USA no. 05072 -AB) and coated with ionic gold (300\AA) in sputter coating unit E-1010 (*Hitachi Japan*) at high vacuum. Thus, processed samples were scanned under scanning electron microscope (SEM) (S3400N*Hitachi Japan*) at 15KV and high vacuum (10^{-7} Torr) and scanned pictures were taken in different magnifications.

2.2.3 Tissues (liver, kidney and intestines) preparation for transmission electron microscope (TEM)

Tissue Fixation: For TEM studies tissues were collected from liver, kidney and Intestines of immediately after sacrifice of animal were washed with normal saline and added cold Karnovsky's fixative. After one-hour, tissues were removed and slices were done into 10 mm H x W x L size by scalpel blade [17]. After this, fresh fixative was added to all the samples at 4°C in fixation for overnight.

First Washing: These samples were further washed with working buffer (0.1M SCB) 3X times with 30 minutes interval to remove the excess fixative.

Post fixation: Buffered 2% Osmic acid (Osmium tetroxide OSO_4 CAS: 201816-12-0 *Loba Chemie Bombay* India) was added and kept for 2 hours at 4°C.

Second washing was done with working buffer (0.1M) Sodium cacodylate buffer and washed 3X times with 30mts intervals to remove excess post fixative.

Dehydration: All the samples were dehydrated in ascending order of Ethanol (Ethyl alcohol 100%: Hayman Group Ltd., UK F204325) from 30%,50%,70%,80%,90% and kept for 30minutes intervals 100% ethanol keep for one hour at 4°C, again added 100% ethanol and kept one hour at room temperature for complete dehydration.

Clearing: Thus, dehydrated samples were added with propylene oxide (Taab Laboratories Equipment Ltd., England UK, Batch no.28190) and kept at room temperature for 2 hours.

Infiltration: The sections were infiltrated in tandem with a resin mixture (EPOXY resin mixture: Araldite CY212 E006 Taab Laboratories Equipment Ltd., England UK, Batch no. 62930. Hardener : DDSA Dodeceny Succinic Anhydrate Taab Laboratories Equipment Ltd., England UK, Batch no. 62670 and Catalyst: DMP 30: 2,4,6 Tri Dimethyl Amino Methyl)Phenol Taab Laboratories Equipment Ltd., England UK, Batch no. 62670) with three chemicals of resin in a ratio of 5 : 5 : 0.1 by w/w (Sartorius analytical balance, Model 6110). This resin mixture with propylene oxide in the ratio of 1:3, 2:2, 3:1 v/v was used for embedding the samples and kept for 2 hours at room temperature. After this procedure, pure resin was added to the samples kept for 2 hours at room temperature.

Embedding: Thus, resin mixed tissues were inserted in to plastic beam capsules 1cm size (Polypropylene BEEM capsules No. 21600 Ladd Research Industries USA) filled with fresh resin in all capsules.

Polymerization: Embedded beam capsules were dried in a vacuum oven for 2 days at 50°C for first day and 60°C second day to allow for setting.

Sectioning: After removing blocks from vials, samples were sectioned by using ultramicrotome (*Leica ultracut* UCT-GA D/E-1600) which contain glass or diamond knife. The cut sections (60-70nm) were collected, placed on a copper grid (200 mesh) and viewed under microscope.

Staining: Sections were stained with *uranyl acetate* and *lead citrate* used to increase the contrast between different structures in the samples.

Analyzing: Sections were analyzed by Hitachi H-7500 Transmission Electron Microscope (TEM) at 60-80KV under high vacuum (10^{-7} Torr) results were saved as per required magnifications [18].

3. RESULTS

In the Experiment the animal's body weight did not match with the food intake in both control and treated group rats. The treated group rats weighed significantly less 12% decrease in the weight of the as compared to the controls. Hemoglobin concentration was reduced significantly in *Aspergillus* spp., inoculated rice (treated group) when compared to control. Different parameters on bodyweight and haemoglobin levels for control and treated rats are given in Table 1 and Table 2 respectively.

Table 1. Different parameters of the control rats fed on normal rice flour diet

Rat no.	Diet Initial In gms	Body weight in gms Initial	Diet final In gms	Body weight Final in gms	Hb% Initial	Hb in % Final
1	10.4	115	12	220	12.8	14.9
2	9.5	125	14	241	14.6	15.2
3	9.5	117	12.5	230	15.4	14.2
4	10.5	119	12.4	221	14.3	15.0
5	10.65	120	12.8	220	12.8	15.0
6	12	121	14	225	14.3	14.8

Table 2. Different parameters of the treated rats fed on diet with fungus inoculated with rice flour 10^7 *Aspergillus* spp, spores/ml

Rat no.	Diet Initial gms	Body weight Initial gms	Diet final gms	Body weight Final gms	Hb in % Initial	Hb in % Final
1	11.0	119	10	160	14.0	14.2
2	10.5	105	9	172	14.4	14.2
3	10.2	110	8	159	14.8	12.0
4	10.8	120	9.5	155	15.8	13.0
5	10.5	119	9.5	139	14.8	12.8
6	11.5	115	8.5	140	14.8	13.9

On the other hand, serum protein was decreased. Serum calcium, and phosphorous concentrations showed similar changes as hemoglobin showed 12% and 8% decrease in treated and controls respectively. Alkaline phosphatase concentration was increased by 35-40%. No mortality was recorded in experimental groups within 28 days. This may be due to adaptability of biological system to varied conditions, even though there was a change in the diet composition.

3.1 Ultrastructure of Liver

Light microscope study: Cell infiltrations in the parenchymatous hepatocytes clearly showed in the cytoplasm of treated rat livers by Optical microscope as compared to control rats. In control rats there was no parenchymal damage and inflammation in rats fed with *Aspergillus* spp., inoculated rice.

Electron Microscope study: In the Scanning Electron Microscope (SEM) studies, morphological changes were observed in the surface as well as transverse section (T.S) of liver of treated group (Fig. 8) when compared with control rat liver (Fig. 7). Ultrastructural changes showed by Transmission Electron Microscope (TEM) that hepatocytes were clearly observed in treated group rat livers as compared with control rat group. Particularly, glycogen clumps were observed in the cells of liver hepatocytes. Excessive development of smooth endoplasmic reticulum (SER) and swollen mitochondria, abnormalities with discontinued fragmented membranes in the boundary of internal layers of mitochondria were other abnormalities observed in treated group rats. Thus, severe damage of mitochondria occurred in rats fed with *Aspergillus* spp., inoculated rice. In the cytoplasm of treated rat liver Golgi bodies were well developed, increase of secondary lysosomes with multivesicular bodies apoptotic bodies like cytoplasm organelles were observed as degenerated. Phagosomes were surrounded in Kupffers cells as shown by TEM study in the treated rat liver cells. In the control rat liver these abnormalities were not observed.

3.2 Ultrastructure of Kidney

Light microscope studies showed that kidney of treated group rat (Fig. 4) patch like mononuclear cell infiltrations in the cortical region of prominent necrosis of proximal tubules, degraded renal tubules and densely eosinophilic apoptotic cells

in the proximal tubule epithelium were observed in the treated group rat kidney but it was absent in control rat kidney (Fig. 5). The SEM studies showed that surface morphological changes were not clear in treated group rat kidney (Fig. 10). However, the transverse section of the kidney showed the changes in the glomerulus part of kidney in treated groups rats when compared to control rats kidney (Fig. 9). TEM micrograph of the control group showed normal in appearance (Fig.15). The kidneys of rats fed with *Aspergillus* sp., diet had large amounts of interstitial tissue with patch like mononuclear cell infiltrations particularly around the blood vessels when compared to treated group rats (Fig.16). Loss of cytoplasmic content was observed in some of the renal tubule cells and degradation of the cell progresses, nuclear changes in treated group. Also, observation of many apoptotic cells and apoptotic bodies and large tubule epithelial cells containing small cytoplasmic vacuoles indicating cellular swelling. This resulted from a shift of extracellular water in to the cell. Prominent necrosis of proximal tubules was also seen, demonstrated by increased cytoplasmic eosinophilia and disappearance of cellular outlines and nuclei. Moreover, areas of debris and possibly from necrotic parenchyma components were seen near the inflammatory cell infiltrations in the renal cortex of treated group rat Kidney.

3.3 Ultrastructure of Intestines

Light microscopy studies showed that normal epithelial cells in the inner wall intestines of control rats (Fig. 5) but little damage of epithelium in the inner surface of treated group rat intestines were observed. Also, accumulation of fluids was observed in the treated group rats. (Fig. 6). In control group (Fig. 5) the intestinal villi were very clear intestines luminal surface these cells for absorption and also called as intestinal fingers projections of mucosa. The luminal surface of the Intestines finger like projections of mucosa (intestinal villi) were observed. Intestines of *Aspergillus* spp., fungus inoculated fed rats showed the damage micro villi structures observation of damaged inner epithelium in the later stages caused less absorption of food and these changes led the complete damage of intestines and finally death in the treated rats.

In the SEM studies showed covering the villi, the loose connective tissue (Lamina propria) was beneath the surface epithelium. The intestinal epithelium detached from the lamina propria

which covering of the villi forming center of the villi in the treated group rat intestines. Prominent interstitial edema was found in the lamina propria. In addition, some parts of intestinal villi were damaged and the internal spaces between

the crypts of Lieberkühn were also observed in treated group rat intestines (Fig. 10) while it was absent in the intestines of the control rats (Fig. 11).

HISTOPATHOLOGY STUDY

Liver

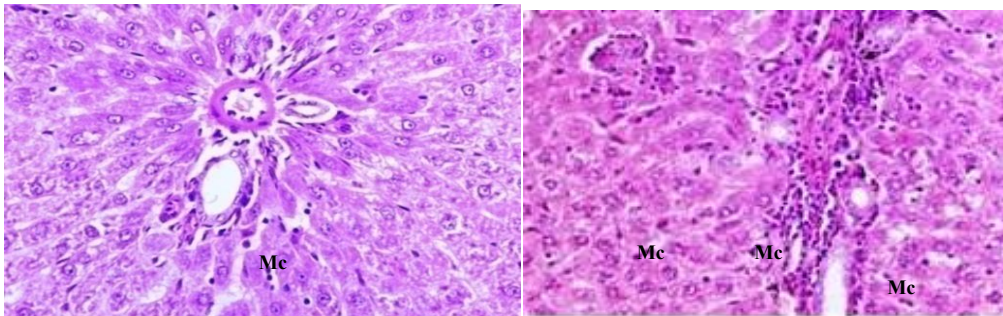


Fig. 1. (Control) Barr=25 um Fig. 2. (Treated) Barr-25 um

Kidney

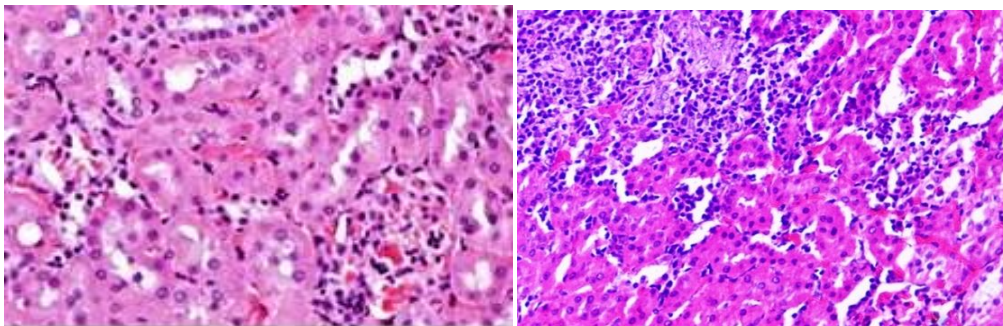


Fig. 3. (Control) Barr=20 um Fig. 4. (Treated) Barr-25 um

Intestines

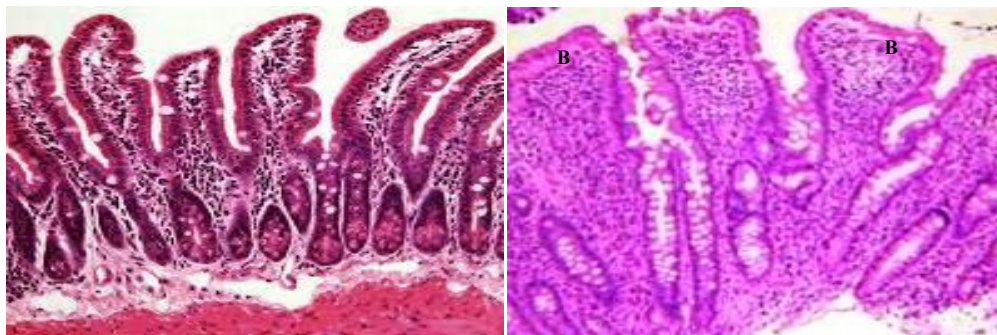


Fig. 5. (Control) barr-20 um

Fig. 6. (Treated) Barr=25 um

Plate 1. Histology of Rat Liver (n=6) showing normal Hepatocytes with portal tract (Fig. 1 Control) normal hepatocytes with inflammation in portal tract Mc Mitotic cells (Fig. 2), Kidney showing normal glomeruli with tubules in control (Fig. 3) inflammation with loss of tubules (Fig. 4) and duodenum showing normal villus and inflammatory cells in Control (Fig. 5) and moderate blunt B villus with inflammation observed in light microscope studies

SCANNING ELECTRON MICROSCOPE STUDIES

Liver

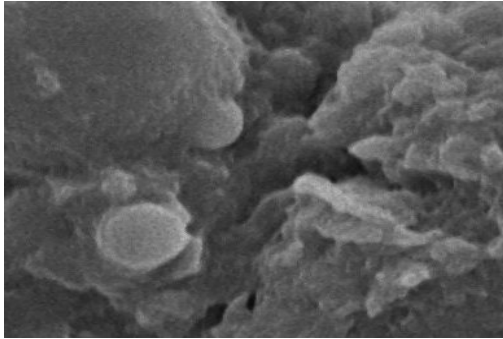


Fig. 7. (Control)

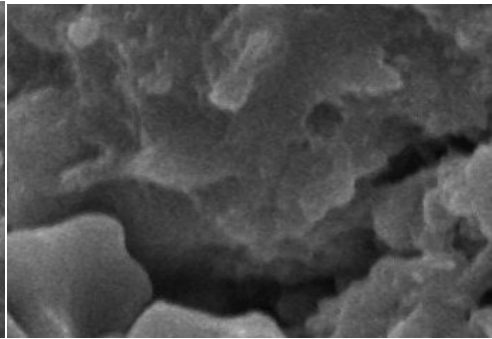


Fig. 8. (Treated)

Kidney

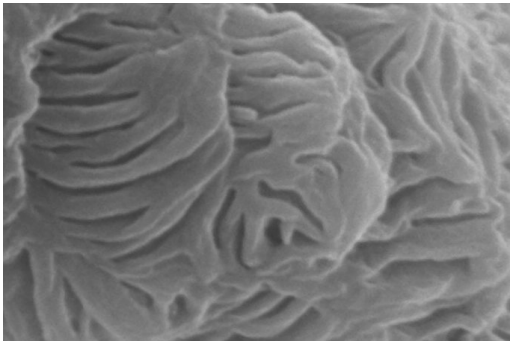


Fig. 9. (Control)

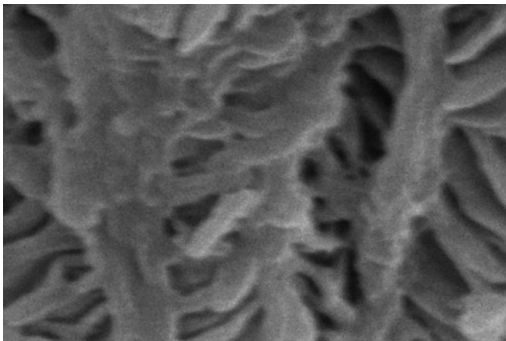


Fig. 10. (Treated)

Intestine

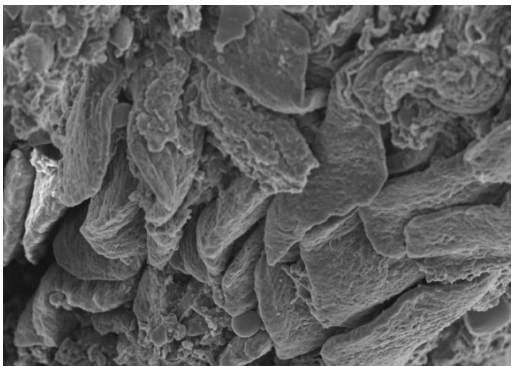


Fig. 11. (Control)

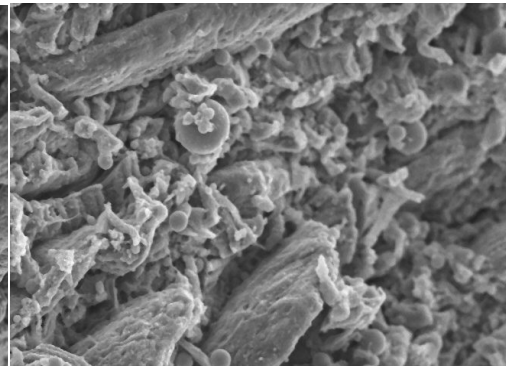


Fig. 12. (Treated)

Plate 2. Scanning Electron Micrograph of Rat Liver control (n=6) showing normal Hepatocytes with portal tract nuclei are spherical (Fig. 7) Mag x120 Normal hepatocytes with inflammation in portal tract and enucleated concavity in treated Rat liver (Fig. 8) Mag x120, Kidney showing normal glomeruli with blood capillary cells in control (Fig. 9) Mag x170 inflammation with loss of tubules with Abnormal capillary tubules (Fig. 10) Mag x170 and Intestines control showing normal villus and inflammatory cells (Fig. 11) Mag x 300 and Intestines treated showing moderate bluntly villus with inflammation observed in treated Rat (Fig. 12) Mag x300

TRANSMISSION ELECTRON MICROSCOPE STUDIES OF RAT LIVER

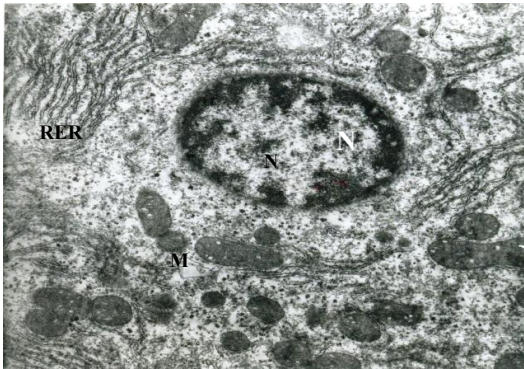


Fig. 13.

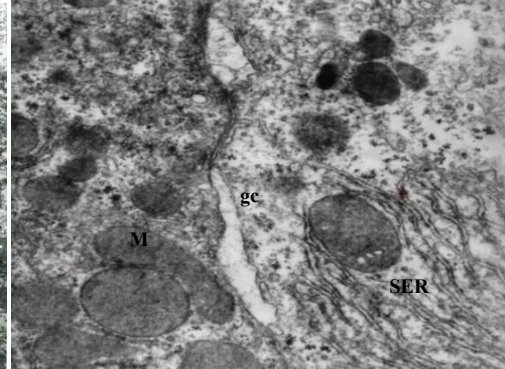


Fig. 14.

KIDNEY

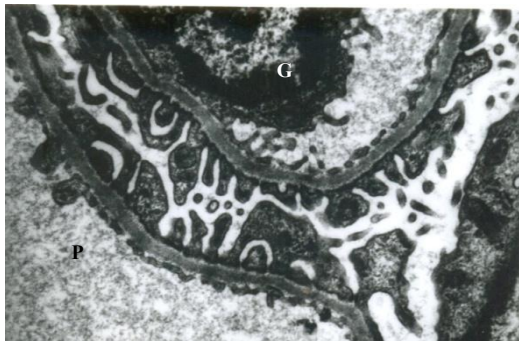


Fig. 15.

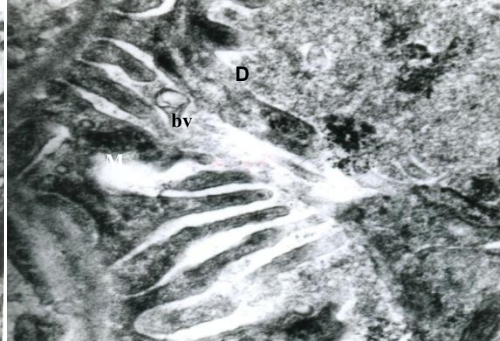


Fig. 16.

INTESTINE

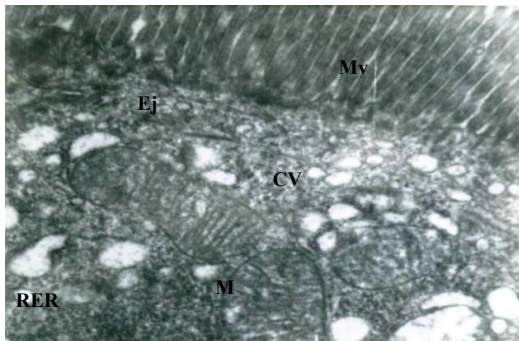


Fig. 17.

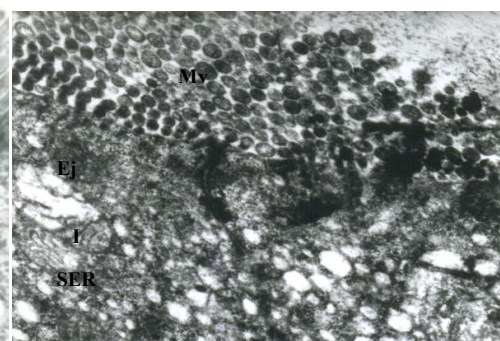


Fig. 18.

Plate 3 Transmission Electron Micrograph of Hepatocyte from Rat Liver control (n=6) N,nucleus Nc, Nucleolus; M, mitochondrion; RER rough endoplasmic reticulum; gc, glycogen clumps Bar=300nm (Fig. 13 Control),normal hepatocytes with inflammation in portal tract Bar=300nm (Fig. 14 Treated), Kidney showing normal G Glomeruli with tubules in control, P proximal tubule. (Fig. 15) I inflammation, Patch like mono nuclear cell infiltration by Blood vessels, D debris (Fig. 16) and Intestines showing normal villus and inflammatory cells in control, Cv cytoplasmic vesicles Mv Microvilli; Ej end junction (Fig. 17) and moderate blunt villus with inflammation observed in treated. (Fig. 18)

Transmission Electron microscope studies also showed cellular damage including inner walls (Lumen) of the intestinal epithelium in the treated group rats. No such changes were observed when compared to control rats (Fig. 17). Histologically the intestines (small) contains 4 layers in which mucosa and submucosa combinedly called as plicae which are folded like structures to increase absorption in the lumen. Epithelium of inner layer contains enterocytes, goblet cells and crypts of Lieberkühn in which the enterocytes are lengthy containing mainly absorptive cells, goblet cells which secretes mucin. Crypts of Lieberkühn are large cells which contains stem cells.

The Intestines of the treated rats showed the histopathological changes in the crypt of villi where Paneth cells containing secretory granules, stem cells, enterocytes and mucin secret goblet cells. In the small intestine crypts of Lieberkühn which are considered as important defensive mechanism against infection of *Aspergillus* sp. The crypts of Lieberkühn showed erosion in the wall of treated intestines which contains stem cells. These stem cells are very important to give rise enterocytes goblet cells and entero-endocrine cells. The damages were clearly observed in terminal cells as the surface the villi was damaged and also effected the release of stem cells and entero-endocrine cells in the treated villi (Fig. 6)

Pathogen protect 'Paneth' cells which produces protective agents' lysozymes, defensins and Peyer's patches in Ileum contain MALT (Mucosal Associated lymphatic tissue) cells are antibody producing cells to protect from the infection in the intestines. Structural differences were observed in the treated Rats and also cytoplasm in the luminal surface web zone. Terminal cell organelles were observed beneath the microvilli. Below the ending junction of cytoplasm, branched profile of endoplasmic reticulum was also observed in the intestines of rats fed with *Aspergillus* spp., inoculated rice. These were absent in the control rats. Mitochondria is flattened and swollen in the epithelium of intestines showing rod shaped filamentous organelle distributed in the cytoplasm of cells. Also, mitochondria were in spongiform appearance containing electron-lucent materials. Further highly expanded endoplasmic reticulum with abundant of cytoplasm vesicles in the epithelial cells in the treated group of rats intestines was observed.

Branched profiles of smooth endoplasmic reticulum (SER) were abundant in the cytoplasm below the end junction of epithelium. Mitochondria is rod shaped, filamentous distributed in the cytoplasm. In the intestines of treated group (Fig. 18). Control group rats intestines showed barring access of Juxta luminal junctional complexes (tight junctions) to the intercellular clefts from lumen intact were observed (Fig. 17). But in treated group, rats showed junctional complexes between the absorptive cells lining the intestines opened and detached in the intestinal surface area. Also, in treated group rats, intestine cytoplasmic swelling in the epithelial cells and spaces between epithelial cells number of eosinophilic granulocytes and mast cells were also observed in the lamina propria of treated group rats.

Thus, *Aspergillus* spp., infection effected the intestinal cells and inner layers of mucosa and submucosal cells and finally effected the absorption surface the absorption of food from the intestines.

4. DISCUSSION

In the present study the histopathological effects of *Aspergillus* spp., on rat liver, kidney and Intestine were investigated using light and electron microscopy. In the liver of treated rats, hepatomegaly was observed and this condition could be associated with disturbances in the liver enzymes, metabolic activities and physiology including drug and fat metabolism were attributed to accumulation of mycotoxins [19]. Nuclear and cytoplasmic pleomorphism was observed in the hepatocytes from the treated group. Necrotic cells showed an increase in eosinophilia that was attributable, in part to increased binding of eosin to denatured intra cytoplasmic proteins and condensing of nuclei (pyknotic nuclei). Dense eosinophilic hepatocytes with pyknotic nuclei were also seen in the liver of treated group rat diet suggested that *Aspergillus* spp., induced hepatic cell death [20] Ultrastructural findings, such as swelling of the mitochondria, discontinuity of organelle membranes and breakdown of cell organelles, were also indicative of hepatocellular necrosis. Mitochondria were appeared to be important targets to all types of injuries such as mycotoxin induced stimuli. Also, swelling in mitochondria was observed under toxic conditions. Mammalian cells are dependent on oxidative metabolism for long term survival. In the present studies irreparable damage to mitochondria in hepatic

cells caused apoptosis and cell components progressively degraded. This had resulted in foci of cellular debris and mononuclear cell infiltration resulted in hepatic tissue inflammation, could be interpreted as a response to fungus-induced liver damage in the treated group [21]. Presence of these structures was taken as a sign of increased cellular digestion of food-borne toxic material, or damaged organelles. Well developed Golgi organelles were seen, and this was unsurprising because they influence lysosome formation. Many apoptotic bodies were also observed in the hepatocytes and Kupffer's cells of rats in the treated group. It is noted that characteristic features of apoptosis are condensation of the chromatin to the periphery of the nucleus, shrinkage of the cytoplasm and formation of apoptotic bodies, which can be phagocytosed by macrophages.

Apoptotic bodies within Kupffer's cells were also observed in the present study (a component of the diffuse mononuclear phagocyte system). It is also observed that mitochondrial damage can also trigger apoptosis as cytochrome c via an integral component of the electron transport system [22] and some mitotic fibers in the liver of rats in the treated group indicating that hepatocellular proliferation may occur in an attempt to replace necrotic and apoptotic liver cells. Necrosis in the hepatic tissue could be due to the clearance and reduction of glutathione transferase, an antioxidant enzyme plays a vital role in detoxification process for removal toxic metabolites that have been formed by *Aspergillus* spp., in the experimental rats.

Transmission electron microscope (TEM) studies of rat kidneys in treated group with *Aspergillus* sp., indicated the formation of small clear vacuoles and water accumulation in the cells [23]. Also, the swelling in the kidney cells with formation of cytoplasmic vacuoles with large tubules in epithelial cells were observed and this condition was attributed to the improper sodium-potassium shunt mechanism between the cells. Other observations were, increased in the number of cells of eosinophilia in the proximal tubules and formation of necrotic cells in cytoplasm. These pathological changes in the kidney resulted in increased eosin binding capacity to intracytoplasmic proteins. Also, mycotoxins of *Aspergillus* spp., induced the renal tubule proliferations indicating the damage in the kidneys as observed by presence of apoptotic

cells and necrotic tissues in the kidneys of treated group rats [24,25].

The pathological changes were observed in cytoskeletal elements including intercellular junctions in the intestinal epithelium of the treated rats. These changes were attributed to increase in calcium levels due to the toxic effects of *Aspergillus* spp., in mitochondria of intestines thus effected function of the gut. In the rats treated group the smooth surface of endoplasmic reticulum indicated lack of development in ribosomes which are the main sites of protein synthesis. Protein synthesis is very important for formation of muscles, maintenance of muscles, development of enzymes and development of defensive mechanisms including antibodies. Also, the reduction in the number of mitochondria in the cells of the visceral organs reduced the energy release and also utilization of energy in treated rats. Formation of improper cristae in mitochondria with sparsely developed intestinal villi could be attributed to poor digestion and absorption of nutrients in treated rats.

In the treatment group, the rats showed mitochondria were electron lucent, spongiform appearance in the epithelial cell suggesting that mitochondria were swollen in toxic conditions of *Aspergillus* sp., In the treated group rats, damage in intestinal mitochondria was also observed. This damage resulted in expansion of terminal part of rough endoplasmic reticulum in intestinal crypt resulted in accumulation of lipids or lipoproteins [26]. This could be due to micro vesicular changes and fat deposition in rough and smooth endoplasmic reticulum [27]. Also, in treated the smooth endoplasmic reticulum synthesizes triglycerides which contains essential enzymes may act on monoglycerides and fatty acids. The accumulation of triglycerides in the treated is a risk factor for degenerative chronic diseases. In the lumen of Intestinal epithelium, swollen cytoplasmic cellular damage was due to mycotoxins of *Aspergillus* spp., and other toxic substances, resulted in accumulation of water in extra cellular boundaries. Thus, abnormal condition in sodium and potassium shunt mechanism at the cellular membrane resulted in water accumulation in the cells of the treated group rats intestines.

In general, vascular permeability induces the cause of intestinal edema when histamine released from mast cells so that similarly rise in the number of eosinophilic granulocytes. Mast cells are observed in allergic and toxic

Table 3. Serum calcium (mg/dl), phosphorous (mg/dl) and alkaline phosphatase

Sl.No.	Group	Weight Gain(gms)	Serum Calcium mg/dl		Serum Phosphorous mg/dl	Alkaline Phosphatase U/L
			Before	After		
1.	Control n=6	56.8±2.0	5.9±0.23	9.5±0.35	9.8±0.52	90.56±18.35
2.	Fungus infected n=6	26.8±0.89	6.25±0.31	6.3±0.21	8.3±0.11	154.5±7.46

Values are mean ±SEM Variation in superscripts between means values for given parameters indicates significant differences (Anova)(P<0.05). Difference of significance greater than 5% are mentioned appropriately in the text. For serum calcium the comparison before and after the treatment period was done by paired t test.

Group Control –NS Group Fungus infected –P<0.05 NS not significant

conditions. In this study the findings of intestinal damages like eosinophilic granulocytes and mast cells in lamina propria and enlarged intercellular vacuoles in the epithelium of lumen of treated rats group was due to the damage caused by mycotoxins of *Aspergillus* spp [28]. Also, the infection caused all forms of cellular damages in the cells of visceral organs and induced lipid peroxidation by generating free radicals that led to cellular damages [29]. Further, oxidation of antioxidants by mycotoxins led to reduction in the concentrations of antioxidants in the animal body.

There were no major changes at the biochemical level, in the intestine of treated groups when compared to control (Table 3). There were no difference in biochemical parameters like hemoglobin, serum protein level and body weights between treated and control rats. This may be due to adaptability and sustainability of biological system to varied conditions, even though there is a change in the diet pattern. The results of this study thus indicated the harmful effects of diet contaminated with *Aspergillus* spp.

5. CONCLUSION

It was concluded that when the rats were fed on rice inoculated with *Aspergillus* sp., caused inflammation and cellular damage in liver, intestinal tissue and kidney. Further, the infection also caused inflammation and damage to the glomerulus tubules in kidney. Also, Ultrastructural damages were also observed particularly in Intestinal morphology including villi led to seize the absorption of food in the rat intestines. The results in the present findings warrant further investigations of the role of *Aspergillus* sp., in the etiology of human and animal diseases that occur frequently in regions of cereal grain production and human consumption. However, this was the first

microscopical study showing pathological changes in rats after ingestion *Aspergillus* spp., fungus inoculated rice and its effects on liver, kidney and intestines function in rat model. Detection of fungus at an early stage rice kernel will be useful for to avoid consumption of rice from the godowns without checking further for quality control of the rice grains for human consumption.

ETHICAL APPROVAL

The research study was reviewed and approved by Scientific Advisory Committee (SAC). Using of animals in this study was approved by the Institutes animal ethics committee (P-9754/IAEC/NIN/2013-14/NHS/WNIN/RATS) of ICMR-National Institute of Nutrition, Hyderabad, India. The approval ensures the guidelines set by the Government of India in this study was strictly implemented and followed.

ACKNOWLEDGEMENTS

Thanks are due to Dr. Hemalatha Rajkumar, Director & Scientist G, ICMR-National Institute of Nutrition for constant support to complete this project by providing lab facilities and funds for this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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