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METANIL YELLOW - AN AZO DYE INDUCED HISTOPATHOLOGICAL AND ULTRASTRUCTURAL CHANGES IN ALBINO RAT (*RATTUS NORVEGICUS*)

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ABSTRACT
The azo dye Metanil Yellow is used in different food items for dyeing and colouring purposes. The chronic exposure of this non-permitted food colour as well as additive in albino rat (*Rattus norvegicus*) for 30 days at a dose of 3.0g/kg body weight was invested through histopathological and ultrastructural changes in stomach, intestine, liver and kidney. Several changes were found under histopathological as well as ultrastructural observations. Histopathological lesions as observed in stomach were disruption of gastric folds, profuse secretion of mucus, necrosis in columnar epithelial cells and gastric glands. In intestine, the villi were damaged severely. The absorptive columnar epithelial cells were totally damaged in some regions, brush border and lamina propria were disrupted due to toxicity. Histopathological lesions were also observed in the liver and kidney of albino rats. In liver, there was an extensive degeneration of hepatocytes, diminish in cytoplasmic content. Appearance of pycnosis of nuclei and damage occurred in the central vein regions. In kidney, necrosis of tubular epithelium, cloudy swelling of epithelial cells of renal tubules and disruption in Bowman's capsule were also prominent features of toxicosis. Under ultramicroscopic observations lesions were found in the mucosal folds and columnar epithelial cells of stomach and intestine. Several changes were also shown in the absorptive columnar epithelial cells as well as in the microvilli of intestine. All these alterations marked the toxic effects of Metanil Yellow in rat.

INTRODUCTION

Different azo dyes are widely used in textile, printing, cosmetic, drug and food processing industries. Metanil Yellow, a monosodium salt of 3-[[4-(Phenylamino) phenyl] azo] benzenesulfonic acid is one among those. It is a non-permitted food colour as well as an additive. Azo dyes are also used in laboratories as biological indicators or as pH indicators. There are varieties of food additives, constituting more than 25,000 items used as preservatives, dye or enhance food quality (Hirschbruch and Torres, 1998; Toledo, 1999). The great bulk of artificial colourings used in foods are synthetic food dye have been suspected of being toxic or carcinogenic and many have been banned whenever possible. Many authors (Reyes *et al*., 1996; Tanaka, 2005; Zraly *et al*., 2006) studied the metabolic and toxicological disorders induced by the administration of specific food colourant additives to rats and other mammals. Many azo compounds are genotoxic in short-term tests and carcinogenic in laboratory animals (Combes and Haveland-Smith, 1982; Sasaki *et al*., 2002). The safety of common red colour amaranth is currently being reinvestigated in view of its suggested carcinogenic and teratogenic effects (Larson, 1975; Holmberg, 1978). In 2001, Tsuda *et al*., reported that amaranth and allura red induce colon DNA damage at a very low dose in mice. The study of the carcinogenic and mutagenic effects of tartrazine are established by some authors which gives variable results (Jones *et al*., 1964; Patterson and Butler, 1982; Maekawa *et al*., 1987; Borzelleca and Hallagan, 1988; Collins *et al*., 1990; Collins *et al*., 1992; Reyes *et al*., 1996; Koutsogeorgopoulou *et al*., 1998; Walton *et al*., 1999; Sasaki *et al*., 2002). Epidemiological studies of food colour additives are difficult, because exposure cannot be accurately assessed. Thus, risk assessment largely depends on laboratory toxicity studies. Allura red produces evidence of both physical and behavioural toxicity in developing rats (Vorhees *et al*., 1983). Poceau 4R (new Coccine) which is permitted as a food colour in the U.S.; but it is neither mutagenic in *Salmonella* (Fujita and Sasaki, 1993), nor teratogenic in mice (Larson, 1975). It is studied that mammalian azoreductase in the intestinal wall or liver reduced the azo dye to free aromatic amines (Chung and Cerniglia, 1992; Chung *et al*., 1992; Prival *et al*., 1988). Oral administration of Metanil Yellow at 3% (w/w) dose level to adult male albino rats resulted in a remarkable decrease in food intake and body weight gain in both normal protein (NP) and low protein (LP) groups (Singh 1996). Chronic consumption of Metanil Yellow by developing and adult rats affected the brain regional levels (Nagaraja and Desiraju,1993). Metanil Yellow (MY) and Malachite green (MG) have promoted effects on the development of hepatic preneoplastic lesions (Gupta *et al*., 2002). Metanil Yellow has been found to cause testicular damage in gametogenic elements to arrest spermatogenesis in guinea pigs, rats and mice (Khanna and Das, 1991) and to cause alteration in haemopoietic system in rats (Mehrotra *et al*., 1974). Reduction products of azo compounds possess toxic and mutagenic properties (Chung, 1983). Although the basic

mechanism of toxicity of Metanil Yellow after ingestion through gastrointestinal tract is very scanty. Orange II caused no change in the serum and tissue cholesterol content in rats (Singh *et al*., 1987). Malachite Green have been found to cause significant alterations in biochemical parameters in the blood of *Heteropneustes fossilis* and it causes depletion of serum calcium and protein levels and also increases the total cholesterol level in blood of catfish (Srivastava *et al*., 1995b). Metanil Yellow induced responses at cellular and sub-cellular organizations in stomach, intestine, liver and kidney of *Heteropneustes fossilis* (Bloch) is invested by Sarkar and Ghosh (2010). Therefore, the present study is mainly focused on the histopathological as well as microanatomical changes caused due to chronic toxicity of Metanil Yellow on albino rats (*Rattus norvegicus*).

MATERIALS AND METHODS

Four albino rats (*R. norvegicus*) in the age group of 2 - 3 months weighing 100 - 250g were allowed to acclimatize to laboratory conditions for one week. After one week animals were divided into two sets containing two animals in each cage as control and treated for the said purpose. One set of the test animals was then subjected to the exposure of the non-permitted food additive Metanil Yellow at a dose of 3.0g/kg body weight for 30 days. The experimental rats were maintained on pellet diet and the water was supplied *ad libitum*. The weight of the each rat was taken prior to experiment as well as after final exposure of 30 days. Both the control and treated rats were sacrificed under chloroform anaesthesia and the desired tissues namely stomach, intestine, liver and kidney were collected from each set's for Haematoxylin-Eosin and SEM study. For histopathological study the tissues were fixed in Bouins fixative for overnight. Paraffin sections were cut at 4 - 5 micron and stained in Delafield's Haematoxylin-Eosin stain.

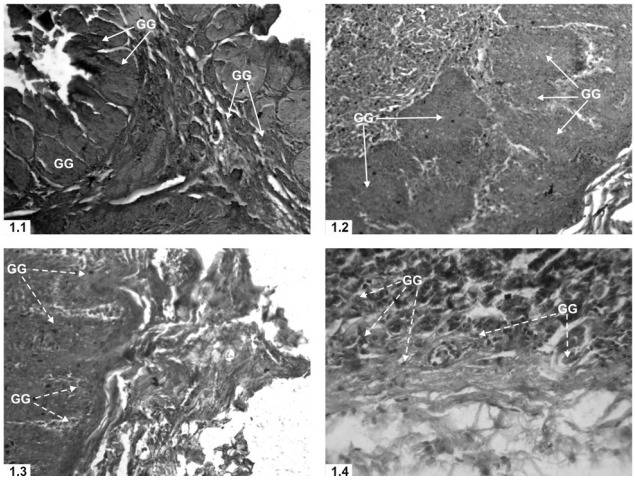
For SEM study the stomach and intestine were incised to expose the mucosal surface and fixed on thin cork sheets. After rinsing it properly in heparinized saline tissues were then fixed in 2.5% gluteraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 24 hours. After fixation, the tissues were washed in buffer, post-fixed for 2 hr in 1 % osmium tetroxide in 0.1 M cacodylate buffer and then dehydrated in graded acetones, followed by amyl acetate and dried by critical point drying method with liquid carbon monoxide. The tissues were cemented to metal stub and coated with gold to a thickness of approximately 20nm. The tissues were examined under Hitachi S – 530 SEM.

RESULTS

Stomach

Histological changes: Stomach is consisted of three regions: the cardiac region, the body, the major part, and the pyloric region, which is the distal part near the pylorus. It is composed of four histological layers, the serous, the muscularis, the submucosa and the mucous in that order from outside within. The serosa is the outermost layer which is composed of simple squamous epithelium. The muscularis consists of two layers – outer most layer is composed of longitudinal muscle fibres and inner is composed of connective tissues. The stomach wall contains four tunics. The tunica mucosa is composed of a single layer of columnar epithelial cells containing microvilli. The surface of the epithelium has a layer of secreted mucus. Below the epithelium is a layer of lamina propria. Separating the mucosa from the underlying submucosa is a layer of smooth muscle, the muscularis mucosae. In the mucosa of rat the muscularis mucosae layer is very thin and consists of a few longitudinally arranged smooth muscle fibres. The epithelium of the gastric mucosa is invaginated to form countless, closely spaced gastric pits. These gastric pits were surrounded by the epithelial cells. The gastric mucosal layer is consisted of simple columnar epithelium and tubular gastric glands (Figs. 1.1 and 1.2). The submucosa is a layer of loose fibrous connective tissue containing large blood vessels and nerves and nerve plexuses. The mucosa is the thickest layer. The gastric glands are arranged in parallel to each other. In the cardia, the gastric glands are branched and coiled and lined by mucus-secreting cells.

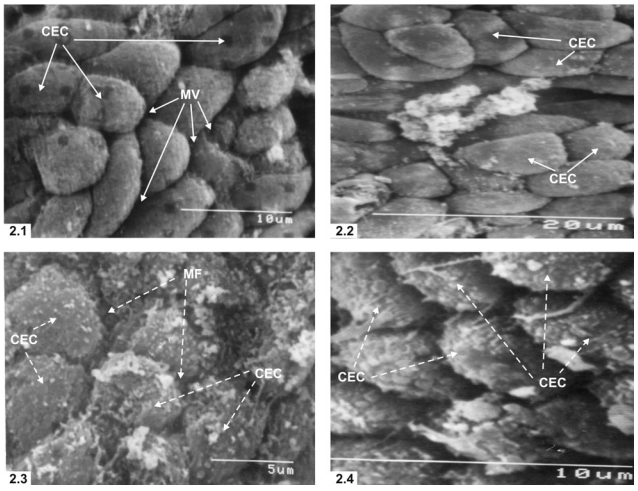
Due to the toxicity of synthetic food colour Metanil Yellow the gastric epithelium was degenerated and excessive secretion of mucus was found over the layer. Necrosis was occurred in the columnar epithelial cells. The erosion was found in the gastric glands (Figs. 1.3 and 1.4) and the degeneration of gastric glands resulting into vacuolation in the tunica propria and submucosa. Muscularis and serosa layers were also damaged after treatment.



Figures 1.1 to 1.4: Changes in stomach histology, 1.1 Normal histological appearance of stomach of control rat showing gastric glands (GG). H&E (10 x 40); 1.2 Control stomach tissue with normal gastric gland (GG). H&E (10 x 100); 1.3 Degenerated gastric gland (GG) caused by Metanil Yellow. H&E (10 x 100); 1.4 Degeneration in gastric gland (GG) caused by the toxicity of Metanil Yellow. H&E (10 x 100)

Ultrastructural changes: The mucosal surface of gastric mucosa is divided into oval and rounded elevations corresponding to the surfaces of columnar epithelial cells which are packed with microvilli (Figs. 2.1 and 2.2). The number of gastric pits are surrounded by gastric epithelium.

The most conspicuous alterations caused by the treatment with Metanil Yellow were the disarrangement of mucosal folds, destruction of epithelial cells (Fig. 2.3), fragmentation and loss of microridges in the apical plasma membrane of the epithelial

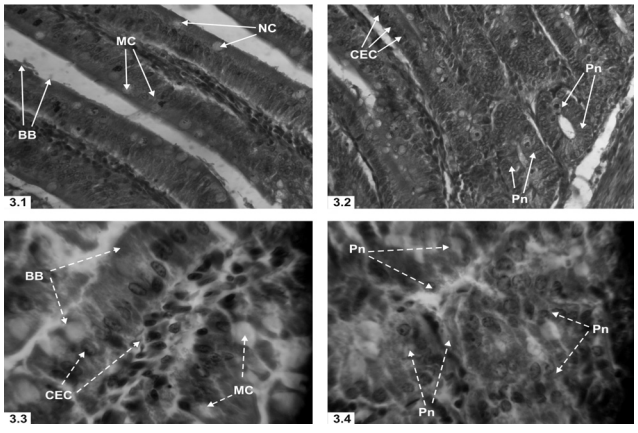


Figures 2.1 to 2.4: Scanning Electron Micrographs of stomach, 2.1 The regulatory arranged mucosal folds supported with oval and rounded columnar epithelial cells (CEC) provided with stubby microvilli (MV) of control rat stomach. (C) x 4000; 2.2 Normal columnar epithelial cells (CEC) of stomach. (C) x 2000; 2.3 Fragmented mucosal folds and necrotic columnar epithelial cells (CEC) of rat stomach due to the toxicosis of Metanil Yellow. (MY) x 6000; 2.4 Necrotic columnar epithelial cells (CEC) of stomach. (MY) x 6000

cells. The arrangement of the columnar epithelial cells (Fig. 2.4) became disrupted showing deep concavities in the structure. Excessive secretion of mucus was observed on some of the epithelial cells. The opening of the gastric pits became the prominent structure as secretory sources of mucus.

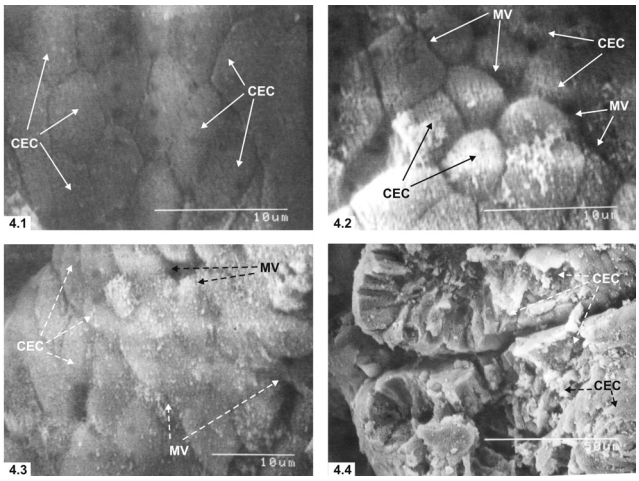
Intestine

Histological changes: The wall of the small intestine consists of the four histological layers. They are also mucosa, submucosa, muscularis and serosa. In case of *R. norvegicus* the mucosa of intestine is made up of simple, long columnar epitheial cells each with basally or centrally placed nucleus. Finger like intestinal villi are projecting into the lumen of the small intestine. The intestinal villi are covered by means of a thin top plate (Fig. 3.1). The mucous cells are interspersed in the intestinal epithelial lining and Goblet cells are interspersed between the absorptive cells (Fig. 3.1). Lamina propria is narrow, long and vascular. The lamina propria of the small intestine is composed of loose connective tissue with blood and lymph vessels, nerve fibres, and smooth muscle cells. The muscularis layer consists of outer longitudinal and inner circular layers of smooth muscles. The microvilli are arranged in rows and in continuation with the microridges of the cell. Paneth's cells are also found in the basal portion of the intestinal glands (Fig.3.2). The packing of the columnar cells is interrupted in certain areas by prominent mucous cells.



Figures 3.1 to 3.4: Changes in intestine histology, 3.1 Normal histological appearance of intestine of control rat showing mucous cells (MC) and columnar epithelial cells (CEC). H&E (10 x 40); 3.2 Control intestine with both normal Paneth's cells (Pn) and columnar epithelial cells (CEC). H&E (10 x 100); 3.3 Degenerated and disrupted brush border (BB) and columnar epithelial cells (CEC) of Metanil Yellow (MY) treated intestine of rat. H&E (10 x 40); 3.4 Metanil Yellow causes degeneration and disruption in Paneth's cells (Pn) and columnar epithelial cells (CEC). H&E (10 x 100)

In *R. norvegicus* the distortion of mucosal epithelium, disruption of connective tissue of lamina propria (Fig. 3.3) were observed. Changes were also found in mucous cell activity and in the epithelial cells. Severe lesions were observed in the top plate and Brush border (Fig. 3.3). Remarkable distortion was recorded in the epithelial cells as well as brush borders (Fig. 3.3) of mucosal epithelium and in certain areas, the lamina propria was severely necrosed. Paneth's cells were also damaged or disrupted (Fig. 3.4) due to the toxicity of Metanil Yellow.



Figures 4.1 to 4.4: Scanning Electron Micrographs of intestine, 4.1 Normal rounded columnar epithelial cells (CEC) of intestine. (C) x 5000; 4.2 Columnar epithelial cells (CEC) with prominent microvilli (MV) of control rat intestine. (C) x 5000; 4.3 Necrosed microvilli and disrupted columnar epithelial cells of MY treated intestine. (MY) x 3000; 4.4 Disrupted columnar epithelial cells (CEC) of Metanil Yellow (MY) treated rat intestine. (MY) x 1000

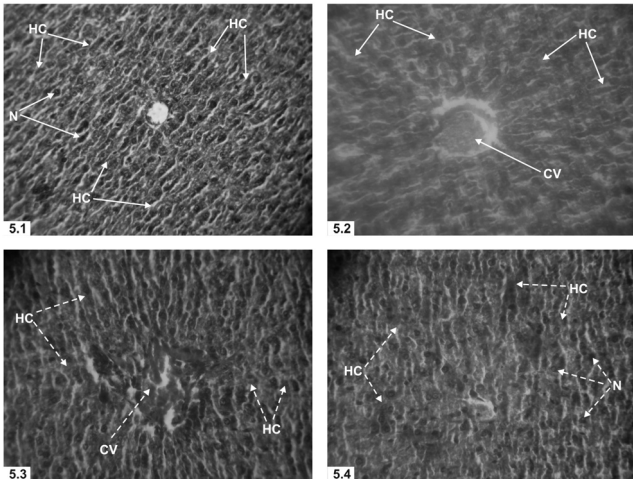
Ultrastructural changes: Intestinal mucosa of *R. norvegicus* was packed with round or columnar epithelial cells. Epithelial cells were provided with densely covered microvilli (Figs. 4.1 and 4.2). The packing of the columnar cells was interrupted in certain areas by prominent mucous cells.

Metanil Yellow intoxication caused serious damage in the mucosal folds. The toxicity loosened the structural configuration (Fig. 4.3) of columnar epitheial cells. Microvilli of the columnar epitheial cells became also disrupted and damaged (Fig. 4.3). Metanil Yellow intoxication showing a number of fragmented secondary mucosal folds in the

concavities between the primary mucosal folds. The columnar epithelial cells loosened their structural configuration (Fig. 4.4). The distinct nature of microvilli of columnar epitheial cells became disrupted heavily (Fig. 4.4).

Liver

Histological changes: The liver is the largest gland of the body. Liver consists of numerous polyhedral hepatic lobules which are formed of a polygonal mass of tissue. Each of these lobules (Fig. 5.1) contains portal spaces at the periphery and a vein, called Central or Centro lobular vein (Fig. 5.1), in the centre. Each lobule is surrounded by groups of three tubes, each group being called a portal triad. The liver cells or hepatocytes (Fig. 5.2) are polyhedral radially disposed and are arranged like the bricks of a wall. These cellular plates are directed from the periphery of the lobule to its centre and anastomose freely, forming a labyrinthine and sponge like structure. The space between these plates contains capillaries, the liver sinusoids. In histological sections, hepatocytes have one, or occasionally two, spherical nuclei (Fig. 5.2) with prominent nucleoli.



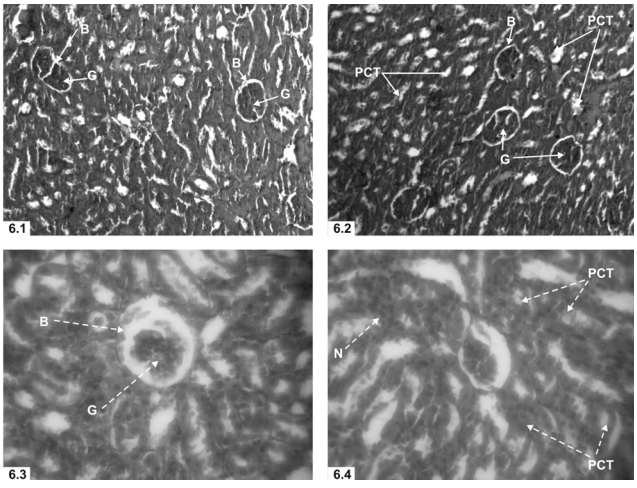
Figures 5.1 to 5.4: Changes in liver histology, 5.1 Normal histological appearance of liver of control rat showing hepatocytes (HC) and nucleus (N). H&E (10 x 10); 5.2 Control liver with both hepatocytes (HC) and central vein (CV). H&E (10 x 40); 5.3 Degenerated and disrupted hepatocytes (HC) and central vein (CV) of Metanil Yellow (MY) treated liver of rat. H&E (10 x 40); 5.4 Metanil Yellow causes degeneration and disruption in hepatocytes (HC) and nucleus (N) of liver of albino rat. H&E (10 x 40)

Administration of Metanil Yellow for 30 days caused huge changes in liver. Necrosis (Fig. 5.3) were found after treatment. Damages were also shown in the central vein region with severe disruption in blood vessels (Fig. 5.3). Liver cells or hepatocytes (Fig. 5.4) were damaged or disrupted after treatment. There was pycnosis in the liver nuclei (Fig. 5.4) were also prominent features of toxicity.

Kidney

Histological changes: The Bowman's capsule is continuous with cuboidal, or low columnar epithelium of proximal convoluted tubules (Fig. 6.1) which is longer than the distal convoluted and contains only three to five spherical nuclei. The distal convoluted tubules (Fig. 6.2) differ from the proximal convoluted tubules because they have no brush border, no apical canaliculi, and smaller cells. Each distal convoluted tubule is joined by a short transitional segment, the collecting tubule, to a collecting duct.

In case of treated condition the parts of the kidney became damaged or disrupted. The brush borders of the proximal convoluted tubule (Figs. 6.3 and 6.4) were ruptured. Several changes were also found in distal convoluted tubule (Fig. 6.3) as well as in collecting tubule. The Bowman's capsule as well as glomerulus (Fig. 6.4) was affected after treatment and vacuolation in the haemopoietic tissues were also shown. Damages were also found in proximal convoluted tubules as well as in distal convoluted tubules (Fig. 6.4).



Figures 6.1 to 6.4: Changes in kidney histology, 6.1 Normal histological appearance of kidney of control rat showing Bowman's capsule (B) and glomerulus (G). H&E (10 x 100); 6.2 Control kidney tissue with normal Bowman's capsule (B), glomerulus (G) and proximal convoluted tubule (PCT). H&E (10 x 100); 6.3 Degenerated Bowman's capsule (B) and glomerulus (G) caused by toxicosis of Metanil Yellow (MY). H&E (10 x 100); 6.4 Degeneration in proximal convoluted tubule (PCT) and nucleus (N) caused by the toxicity of Metanil Yellow. H&E (10 x 100)

DISCUSSION

Intentional addition of substances, chemicals and colourants in food is an age old practice to make them more palatable, enjoyment, or to protect flavours and vitamins and also to preserve it. Actually, colours are used in food for centuries to increase consumer acceptability. Natural colours are more expensive than the synthetic ones. It has been examined that the Indian population consumes 220 mg of food colours per year (Singh, 1997). Dyes and pigments are mostly coloured substances used for colouration. The chemicals used for their synthesis are hazardous for human life. Besides mortality the other adverse effects of Metanil Yellow to the test fishes included loss of body weight, changes in body colours, restlessness and jerky and random movement (Mall and Kishore, 1995). The result of the present histopathological investigation showed that Metanil Yellow toxicity disrupt the gastric epithelium as well as necrosis in the columnar epithelium cells of stomach and there were also some serious distortion in mucosal epithelium and connective tissue of lamina propria of intestine hampering the absorptive process.

The stomach is the dilated part of the digestive system which

connects the oesophagus and small intestine. Its function is to act as a reservoir of food, as well as a mixer and by its peristaltic movements allows the food to mix with the secretions of the organs and gastric glands produce various types of secretions. Under histopathological changes the gastric epithelium concomitantly reduced the protection ability of underlying epithelial cells. Clumping of the nuclei was occurred due to dissociation of the cell membranes of the columnar epithelial cells in the stomach region of the albino rat. Swelling, damage and/or vacoulation, with a tendency to necrotization of the mucosal epithelial cells of the stomach as a diagnostic feature of chronic exposure to Metanil Yellow.

The small intestine is the site of terminal food digestion, nutrient absorption, and endocrine secretion. The processes of digestion are completed in the small intestine, where the nutrients are absorbed by cells of the epithelial lining. The small intestine, where the nutrients are absorbed by cells of the epithelial lining. The small intestine extends from the pylorus to the ileoceacal valve, where it joins the large intestine. The luminal end of the columnar epithelial cells of the intestine of the rat which bears a zone of microvilli as well as brush border were also affected due to the toxicosis of the dye (Metanil Yellow).

The liver has an exocrine function, the secretion of bile, which is conveyed to the intestine by a system of ducts. It produces prothrombin, serum lipoproteins. It performs the important function of storing carbohydrate foods and releasing them into the blood at such times as they are needed by the body – a function which was first described as one of internal secretion. Liver cells are the store house of fats, proteins and vitamins and are also an organ of excretion. Pycnotic nuclei with damaged hepatocytes were found in liver of rat.

The kidney is a compound tubular gland which separates urea and other nitrogenous waste products from the blood. It also maintains the constituents of blood plasma at proper levels, and, it has also an important role in regulating the chemical composition of the extra cellular fluid which bathes the cells and tissues of the body. In case of kidney the PCT (proximal convoluted tubule), DCT (distal convoluted tubule) as well as collecting tubule or ducts were disrupted. Huge changes were found in the nuclei of both PCT and DCT.

Under microanatomical changes the results showed that the disarrangement of mucosal folds and destruction of epithelial cells occurred in stomach due to the toxicity of Metanil Yellow. In case of intestine disruption of mucosal folds and columnar epitheial cells occurred by the chronic exposure of Metanil Yellow for 30 days on albino rats (*Rattus norvegicus*).

The present study shows that Metanil Yellow causing damage to the microridge structure located on the apical portion of the columnar epithelial cells of stomach of *R. norvegicus*. It is reported that Metanil Yellow, the frequently used non-permitted food colour was found to cause toxic methaemoglobinaemia in adult human males 2 - 4h after the consumption of rice coloured with it (Sachdeva *et al*., 1992).

Metanil Yellow was also reported to cause cyanosis (Chandro and Nagaraja, 1987). Direct action of Metanil Yellow or its active metabolites on germ cells was also reported (Venkateshwarlu *et al*., 1997). A number of azo compounds are mutagenic in an assays if chemical reduction or microsomal activation, or both, are induced (Chung and Cerniglia, 1992). The renal changes in rabbit were shown following repeated oral dose of Malachite green (Desciens and Bablet, 1994). Malachite green has been found to be mutagenic in rats and mice; and it causes significant developmental abnormalities in pregnant New Zealand white rabbits (*Oryctolagus cuniculus*) (Meyer and Jorgensen, 1983). Incidence of tumors in lungs, breast and ovary have also been reported from rats exposed to Malachite green (Werth, 1958). Toxic effects were shown by oral administration of untreated (influent) and treated (effluent) textile dye waste water on male reproductive systems of adult swiss albino rats and mice (Suryavathi *et al*., 2005).

Khanna *et al*., 1978 reported that Metanil Yellow were found to cause no pathological changes in any of the body organ except the testes of albino rats after kept on diets containing 0.0, 0.1, 0.5, and 3.0% Metanil Yellow for 90 days. Incidence of significant induction by Metanil Yellow, Orange II and their blend on hepatic xenobiotic metabolizing enzymes in rats have been studied (Ramchandani *et al*., 1994). The pathological lesions caused due to chronic toxicity for an exposure of 30 days with a sub lethal dose of 3g/kg body weight was studied on albino rat (*Rattus norvegicus*) to study the damage in the regions of alimentary canal like stomach and intestine as well as liver and kidney at cellular and sub-cellular levels. It has been found that the stomach and liver were damaged more than the intestine and kidney of albino rat by the toxicosis of food additive Metanil Yellow after an exposure of 30 days.

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