

Research Article

Functional and Cellular Exocrine Pancreatic Dysfunction in Male Mice Following Sub-Chronic Exposure to Melamine and Formaldehyde

Wafaa AM Mohamed* and Shefaa AM El-Mandrawy

Department of Clinical Pathology, Zagazig University, Egypt

*Corresponding author

Wafaa Abdou Mohamed Mohamed, Department of Clinical Pathology, Zagazig University, Zagazig, Egypt, Tel: +201010575308; Email: waffa.clinical@yahoo.com

Submitted: 10 May 2016

Accepted: 01 June 2016

Published: 02 June 2016

ISSN: 2373-9282

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OPEN ACCESS

Keywords

- Melamine
- Formaldehyde
- Pancreatic amylase
- Steatorrhea and pancreas

Abstract

Due to the lack of research papers on this topic; we aimed in this work to highlight the bad effects of melamine with or without formaldehyde on the digestive system of mice model including mal-digestion and mal-absorption. Forty adult male Swiss mice were divided into 4 equal groups. The 1st group was treated orally with distilled water and kept as negative control (C group), the 2nd group received 25 mg MA / kg bw (MA group), the 3rd group orally given 50 mg FA /kg bw (FA group) and the 4th group supplemented with 25 mg MA /kg bw + 50 mg FA /kg bw (MA+FA group) for 60 consecutive days. Selective biochemical tests, exocrine pancreatic enzymes and oxidative stress biomarkers were assessed together with spleen and intestine histopathology. The obtained results demonstrated that either MA or FA treatment resulted in a significant decrease in serum total lipids, glucose, sodium, chloride and pancreatic antioxidant enzymes activities as catalase and reduced glutathione contents. However, serum amylase, lipase and lipid peroxidation were significantly increased. These changes were significantly higher in the combination group followed by melamine then the formaldehyde group. Conclusively, chronic exposure to both melamine and formaldehyde have more adverse effects on the exocrine function of pancreas in mice causing maldigestion and malabsorption through their oxidative stress inducing damage in the pancreatic tissue.

INTRODUCTION

Melamine (MA) is an industrial organic chemical product with high nitrogen content. It added to the pet food and milk it can falsely elevate the apparent protein concentration readings [1]. MA is famously used in the production of plastics, glues, kitchenware, commercial filters, dishware and fabrics [2]. Melamine alone was generally considered to be of low acute toxicity in mammals [3]. The acute toxicity of melamine in rodents is reported with oral lethal doses 50 (LD50s) of 3,100 mg/kg (male rats) and 3,900 mg/kg (male mice) [4].

Recently, many experimental studies mentioned numerous adverse effects following MA consumption in laboratory animals, including humoral immunotoxicity [1], ovarian toxicity [5], bladder carcinogenicity [6], nephrotoxicity [7], neurotoxicity [8] and male reproductive toxicity [9]. MA could cause toxic effects in the gastrointestinal tract and liver in mice during acute and sub-acute toxicity study [10].

Formaldehyde (FA, CH₂O) is flammable, colorless, and polymerized at ambient temperature and pressure, with a pungent odour [11]. Formaldehyde is a common environmental contaminant. Despite enormous efforts to reduce the formaldehyde-related hazards, exposure to FA remains one of the most crucial occupational and environmental health problems. Occupational exposure to FA may occur during manufacturing, processing and during the use of FA containing products, mainly via the dermal and inhalation routes [12].

FA is an irritant compound, which can elicit adverse effects on many body organs. It caused testicular damage and genotoxicity [13], respiratory problems in veterinary students [14], neurotoxicity [15], Embryo toxicity and teratogenicity [16], additionally, it induced gastrointestinal system and urinary system disturbances [17].

Melamine formaldehyde (MF) is a member of the amino resin family. Originally developed in the 1930s and prized

for its toughness, chemical resistance, and relative ease of manufacture, MF is incorporated into a wide variety of products that are still in use today. Familiar products include Formica and melamine dinnerware. Commercial applications including fabric impregnation, adhesives, paints, electrical mouldings, glass-reinforced substrates and engineered wood products. These condensed amino-plastic products are generally stable with low emitted formaldehyde ratio [18]. MF found to have an adverse effect on the liver, kidney and brain in rat [19].

Currently, there is little available information on the effects of MF resin toxicity on the exocrine pancreatic function and intestinal micronutrients absorption. Thus, the purpose of the present work was to investigate the potential effects of MA in the absence and presence of FA on digestive enzyme level in relation to the mechanism of oxidative stress induced pancreatic and intestinal cellular damage.

MATERIAL AND METHODS

Tested compounds and chemicals

Melamine extra pure (99.5% purity, M.W. 126.12, acidity, 0.27 ml N % $C_3H_6N_6$) was purchased from Alpha Chemicals, Mumbai, India. Formaldehyde (40% conc.) was purchased from Al Gomhoria Co Egypt. All other reagents and chemicals used were of analytical grade purchased from (Sigma-Aldrich Co. St. Louis, MO, USA).

Ethics Statement

The experimental procedures were carried out according to the general guidelines of the National Institutes of Health (NIH) for the Care and Use of Laboratory Animals in scientific investigations and approved by the ethics of animal use in research committee (EAURC), Zagazig University.

Animal grouping and experimental design

Forty adult male Swiss mice weighting 20–25 gm were used in this study. They were obtained from the Laboratory Animal farm of the Faculty of Veterinary Medicine of Zagazig University and acclimated to the laboratory environment for two weeks prior to use. The animals were housed in stainless-steel cages, maintained in a 12 h light-dark cycle at a controlled temperature (21–24°C) and relative humidity (50–60%), and given standard diet and water ad libitum throughout the study.

After accommodation period, mice were weighed and randomly distributed into four equal groups each containing 10 mice. Group I (C): Control group received distilled water. Group II (MA): Received 25 mg melamine /kg bw/day [20]. Group III (FA): Received 50 mg formaldehyde /kg bw/day [21]. Group IV (MA+FA): Treated with the melamine and formaldehyde at the previously mentioned dose. These regimens were administered orally once daily by stomach tube for 60 consecutive days.

Sampling

At the termination of the dosing (60 Day), mice were fasted overnight. All mice of each group were weighed then sacrificed via cervical dislocation after light ether anesthesia. Non anti-coagulated blood samples were collected from each mice; 1 ml was taken in a glass tube without EDTA and left for 20 minutes

to coagulate at room temperature, and then centrifuged at 3000 rpm for 10 min to obtain serum samples. Serum samples were kept at -20°C until used for serum amylase, lipase, total lipids, and glucose as well as serum electrolytes levels determination. The whole pancreas tissues were removed immediately after sacrifice and washed in physiological saline, then freezed at -20°C for lipid peroxidation and antioxidant enzymes activities estimation. Small parts of pancreas, small and large intestine were collected and fixed at 10% buffered neutral formalin solution for histopathological examination.

Biochemical tests estimations

Serum total lipids, and glucose were assayed colorimetrically according to the methods described by Frings and Dunn [22], Trinder [23] by using a commercial kits of BioMed-Diagnostic, EGY- CHEM for lab technology, Badr City, Industrial Area Piece 170, 250 Fadan In East of Elrubaki, Egypt using a semi-automated Photometer (5010 V5+, RIELE GmbH & Co, Berlin, Germany). Serum levels of sodium and chloride were determined using flame photometer (Model FP 20 seac, Seag Radim Company, Italy) with specific diagnostic kit (BioMérieux, France) as described by Ali [24].

Digestive enzymes activity assay

Serum amylase and lipase activities were measured using an assay kit from Bio-Assay Systems (Hayward, Calif). Their activities expressed by U/L.

Oxidative stress biomarkers

Tissue Malondialdehyde, reduced glutathione and catalase levels were measured colorimetrically using Diagnostic and Research Reagents of Biodiagnostic, Egypt (CAT. No. ; MD 25 29, GR.No. 25 11 and CA 25 17 respectively) following the method applied by Ohkawa et al., Beutler et al., Aebi respectively [25-27].

Qualitative fecal fat microscopy

Qualitative fecal fat microscopy was assessed by the method of Drummey et al. [28] using a glass slides for fecal microscopy on which a small piece of homogenized stool (5-mm diameter) was placed and mixed with 2 drops of 36% acetic acid, followed by 2 drops of 1% Sudan III stain. The slides were held by hand over a hot plate until bubbles appeared, then quickly removed and reheated 2 additional times. They were examined immediately with a microscope

Histopathological examination

The fixed specimens of the pancreas, small and large intestines were dehydrated in gradual ethanol (70-100%), cleared in xylene, and embedded in paraffin. Five-micron thick paraffin sections were prepared and then routinely stained with hematoxylin and eosin (HE) dyes for histopathological examination [29].

Statistical analysis

Data of the current study was statistically analyzed using the computer program SPSS/PC+2001. The statistical method was one way ANOVA test, followed by Duncan's multiple range test [30]. Data are presented as means plus or minus the standard error. The minimum level of significance was set at $P < 0.05$.

RESULTS

MA and/or FA effects on biochemical parameters

The effect of oral supplementation of MA and/or FA for 60 days on selective biochemical variables including total lipids, glucose and serum electrolytes of adult male Swiss mice under the study was given in Table (1).

With respect to the clinico-biochemical report, both individual and co-exposure to MA and FA evoked a significant ($p < 0.05$) decrease in the mean values of serum total lipids, glucose, sodium and chloride compared to that of the control. The decrease was severer in the combination (MF) group then the MA-exposed animals and finally FA-exposed mice.

MA and/or FA effects on digestive enzymes levels and fecal fat

The effect of consecutive MA and/or FA exposure in adult male Swiss mice for 60 days on serum digestive enzymes levels was demonstrated in table 1, the serum amylase and lipase levels were significantly increased in all exposed groups when compared with the control group ($p < 0.05$). The decrease was severer in the combination group then the MA-exposed animals and finally FA-exposed mice. Based on the results showing in Figure 1, and compared to the negative control, the average percentage of fecal fat exhibited about 5, 3 and 2-fold increases in the MA+FA, MA and FA exposed group respectively (Figure 2).

MA and/or FA effects on lipid peroxidation and antioxidant enzymes activities

In comparison to control mice, the lipid peroxidation, MDA of both individual and co-exposed MA and FA mice showed a significant ($p < 0.05$) increase elsewhere, the pancreatic CAT and GSH levels were greatly ($p < 0.05$) decreased. These changes were more clear in the combination group then the MA-exposed animals and finally FA-exposed mice (Table 2).

Histopathological findings

Light microscopical investigation of the HE stained pancreas paraffin sections in the control group showed normal pancreatic

histoarchitecture with normal exocrine portion, serous acini (S) and endocrine part, islets of Langerhans (I) (Figure 2A). The pancreas of MA administered group revealed histopathological changes of both exocrine and endocrine part of the pancreas represented by marked decrease of islet cells. Noticeable increase of the interacinar connective tissue on the expense of exocrine acini and sever congestion of blood vessels was also detected (Figure 2B). The pancreas of FA administered group showed focal acinar damage was represented by pyknotic nuclei of some acinar cells and decreased acinar diameter. Islets architecture was changed by decreased islets diameter as well as decreasing the number of cells composing pancreatic islets (Figure 2C). The pancreas of MA/ FA administered group resulted in sever pancreatic damage including sever congestion of blood vessels, increase of the interacinar connective tissue with decreasing of the diameter of both pancreatic acinar and islets (Figure 2D).

Light microscopical investigation of the HE stained small intestine paraffin sections in the control group showed normal histological structure with intact mucosa, submucosa, muscularis and serosa (Figure 3A). The small intestine of MA administered group showed distortion of its mucosal architecture, mild to moderate hyperplasia of the lamina propria and submucosa with lymphocytic infiltration (Figure 3B). The small intestine of FA administered group revealed moderate distortion of mucosal architecture and development of subepithelial space. Marked hyperplasia of both lamina propria and submucosa as well as hyperplasia of columnar epithelium are lining intestinal villi (Figure 3C). The small intestine of MA/ FA administered group showed severe degenerative changes of villi was noticed among the group treated by combined substances. Sloughing of villi into intestinal lumen, loss of villi, severe atrophy of villi and distortion of mucosal architecture were detected (Figure 3D). Meanwhile, the HE stained large intestine paraffin sections in the control group showed no histological changes were seen among the tunica of large intestine of control animals (Figure 4A). The large intestine of MA administered group showed moderate sloughing of intestinal mucosa (Figure 4B). Histopathological alterations in the large intestine of FA administered group were severer than that of the previous group. The severity included severe sloughing of intestinal mucosa and lymphocytic infiltration among mucosal

Table 1: Clinico-biochemical variables in adult male Swiss mice orally treated with melamine (MA) (25 mg melamine /kg bw/day) and / or formaldehyde (FA) (50 mg formaldehyde /kg bw/day) for 60 days. The values are expressed as means \pm SE. n = 5.

Parameters	Treatments			
	C	MA	FA	MA + FA
Organic constituents				
Sodium (mmol/L)	135.6 ^a \pm 2.3	98.3 ^c \pm 4.4	112.3 ^b \pm 3.9	85.0 ^d \pm 2.8
Chloride (mmol/L)	90.3 ^a \pm 3.5	68.0 ^c \pm 1.5	77.3 ^b \pm 1.4	62.3 ^c \pm 1.4
In-organic constituents				
Total lipids (mg/dL)	399.6 ^a \pm 6.0	351.1 ^b \pm 9.2	378.0 ^a \pm 6.2	308.3 ^c \pm 4.4
Glucose(mg/dL)	106.3 ^a \pm 3.2	77.6 ^b \pm 5.0	94.3 ^a \pm 2.3	70.0 ^b \pm 7.6
Digestive enzymes				
Amylase (U/L)	355.8 ^c \pm 25.8	612.3 ^b \pm 1.8	567.9 ^b \pm 25.3	806.8 ^a \pm 85.8
Lipase (U/L)	74.6 ^c \pm 4.8	103.0 ^{ab} \pm 4.1	94.0 ^{bc} \pm 3.4	119.4 ^a \pm 9.7

Means within the same row carrying different superscripts are significant at ($p < 0.05$).

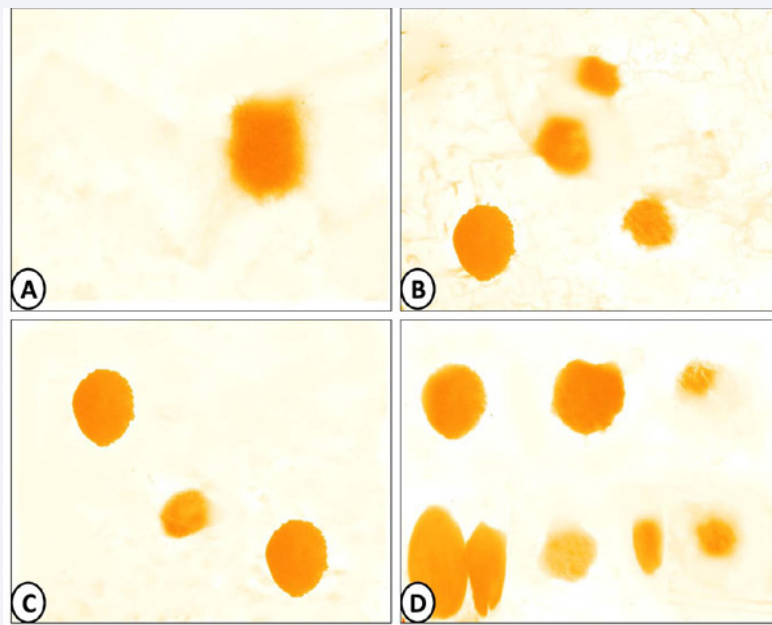


Figure 1 Photomicrographs of 1% Sudan III stained fecal smears showing fat droplets distribution in group C (daily orally 0.1 ml administered distilled water) (A), Group (MA) (25 mg melamine /kg bw/day) (B), Group (FA) (50 mg formaldehyde /kg bw/day) (C) and Group (MA+ FA) (D). (x 200).

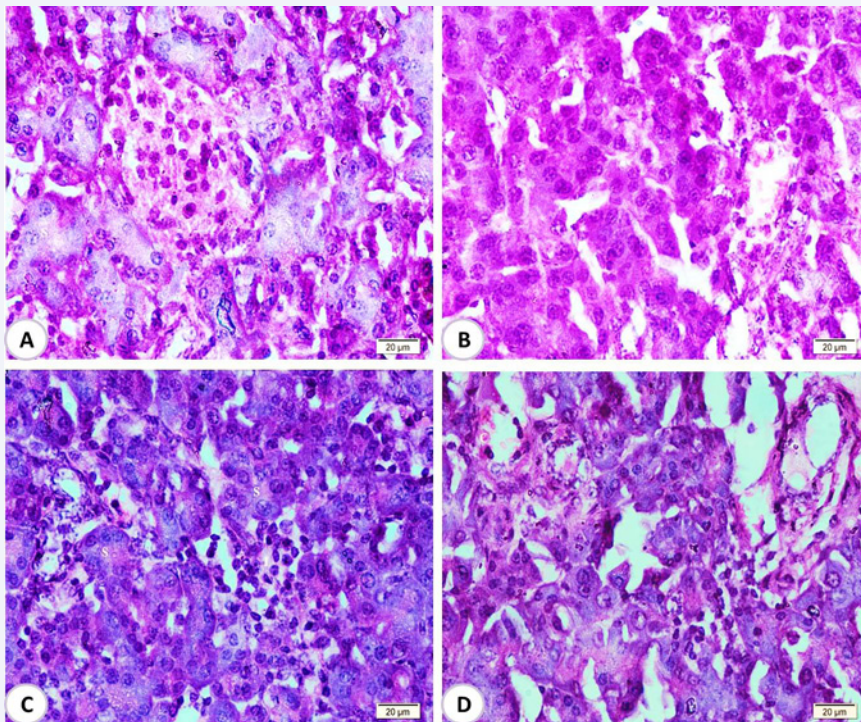


Figure 2 Photomicrographs of the H&E paraffin stained pancreas sections of; (A) group C (daily orally 0.1 ml administered distilled water) showing normal pancreatic histoarchitecture with normal exocrine portion, serous acini (S) and endocrine part, islets of Langerhans (I), (B) Group (MA) (25 mg melamine /kg bw/day) showing histopathological changes of both exocrine and endocrine part of the pancreas represented by marked decrease of islet cells. Noticeable increase of the interacinar connective tissue on the expense of exocrine acini and sever congetion of blood vessels was also detected. (C) Group (FA) (50 mg formaldehyde /kg bw/day) showing focal acinar damage was represented by pyknotic nuclei of some acinar cells and decreased acinar diameter. Islets architecture was changed by decreased islets diameter as well as decreasing the number of cells composing pancreatic islets. (D) Group (MA + FA) showing severe pancreatic damage including sever congestion of blood vessels, increase of the interacinar connective tissue with decreasing of the diameter of both pancreatic acinar and islets.

Table 2: Oxidative stress markers differences in adult male Swiss mice orally treated with melamine (MA) (25 mg melamine /kg bw/day) and / or formaldehyde (FA) (50 mg formaldehyde /kg bw/day) for 60 days. The values are expressed as means \pm SE. n = 5.

Parameters	Treatments			
	C	MA	FA	MA + FA
MDA(nmole/g tissue)	551.2 ^d \pm 64.2	845.0 ^b \pm 31.3	647.8 ^c \pm 52.7	1222.4 ^a \pm 101.9
GSH (nmole/g tissue)	1317.8 ^a \pm 133.8	876.35 ^c \pm 57.5	1103.6 ^b \pm 105.0	424.1 ^d \pm 48.3
CAT (U/g tissue)	216.5 ^a \pm 19.0	197.7 ^b \pm 32.3	205.5 ^b \pm 16.6	139.9 ^c \pm 7.3

Means within the same row carrying different superscripts are significant at (p <0.05).

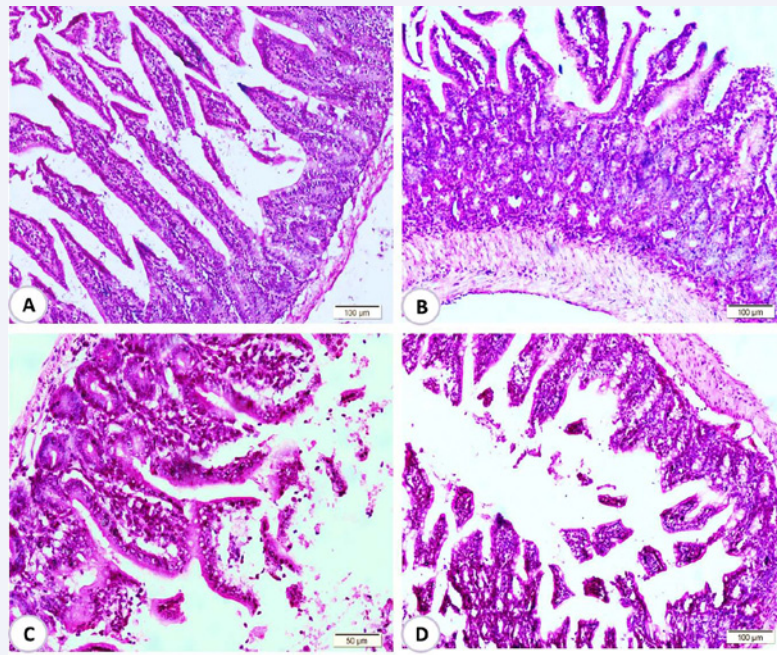


Figure 3 Photomicrographs of the H&E paraffin stained small intestine sections of; (A) group C (daily orally 0.1 ml administered distilled water) showing normal histological structure with intact mucosa, submucosa, muscularis and serosa. (B) Group (MA) (25 mg melamine /kg bw/day) showing distortion of its mucosal architecture, mild to moderate hyperplasia of the lamina propria and submucosa with lymphocytic infiltration. (C) Group (FA) (50 mg formaldehyde /kg bw/day) showing moderate distortion of mucosal architecture and development of subepithelial space. Marked hyperplasia of both lamina propria and submucosa as well as hyperplasia of columnar epithelium are lining intestinal villi. (D) Group (MA+FA) showing severe degenerative changes of villi were noticed among the group treated by combined substances. Sloughing of villi into intestinal lumen, loss of villi, severe atrophy of villi and distortion of mucosal architecture were detected.

propria and tunica submucosa (Figure 4C). The large intestine of MA/ FA administered group showed dysmorphology on large intestine (Figure 4D).

DISCUSSION

The pancreas is a retroperitoneal organ critically important for intestinal digestion. Most of the pancreas consists of the exocrine glands that synthesize and secrete a great majority of digestive enzymes into the pancreatic duct tributaries and on into the duodenum. Pancreatic lipase (PL) is the main enzyme responsible for digestion of dietary triglycerides. PL catalyzes the hydrolysis of 56% of the fatty acids of dietary triglycerides, and gastric lipase, an additional 10% [31]. PL and its related protein PLRP1, a homologous protein of unknown function [32], are secreted by the pancreas and regulated by dietary fat [33]. Alpha-amylase catalyses the hydrolysis of α -(1,4) glycosidic linkages of starch components, glycogen, and various oligosaccharides. A-amylase is a member of glycosyl hydrolase family 13, which

also contains cyclodextrin glucanotransferases (CGTases) and pullulanases [34].

Studies concerning the toxicity of melamine taken orally in humans are nonexistent. Toxicity data mainly come from studies in sheep, cat, dog, mice, and rat. Toxicity can be classified as acute or chronic. The most common toxicity is renal toxicity, which is also the area of most concern to nephrologists [35]. Oral ingestion affects the digestive tract, presenting as nausea, vomiting, and diarrhea [36].

With respect to the digestive enzyme content (Table 1), both individual and co-exposure to MA and FA evoked a decrease in the mean values of serum total lipids, glucose, sodium and chloride which might be due to the exocrine pancreatic insufficiency causing maldigestion related to the toxic effect of both melamine and formaldehyde which represented by steatorrhea and increased fecal fat as found previously by Domínguez-Muñoz [37] who said that exocrine pancreatic insufficiency with steatorrhea

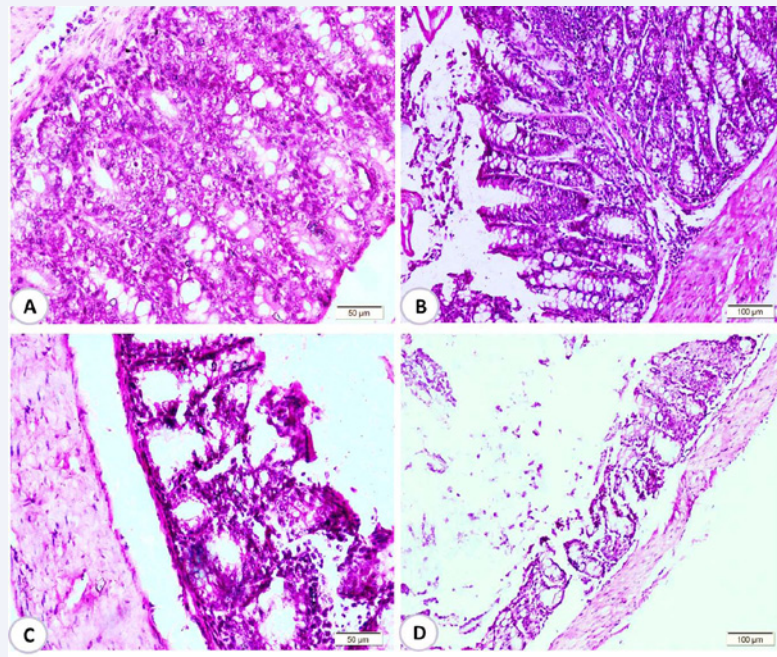


Figure 4 Photomicrographs of the H&E paraffin stained large intestine sections of; (A) group C (daily orally 0.1 ml administered distilled water) showing no histological changes were seen among the tunica of large intestine. (B) Group (MA) (25 mg melamine /kg bw/day) showing moderate sloughing of intestinal mucosa. (C) Group (FA) (50 mg formaldehyde /kg bw/day) showing severe sloughing of intestinal mucosa and lymphocytic infiltration among mucosal propria and tunica submucosa. (D) Group (MA + FA) showing dysmorphism on large intestine.

is a major consequence of chronic pancreatitis. Additionally, a reduction in the secretion of the pancreatic digestive enzymes as lipase and amylase is seen in the duodenum and elevation of their levels in serum as presented in this work. The exocrine pancreatic insufficiency was resulted from pancreatic tissue damage caused by the oxidative stress induced by both substances as previously obtained by Zararsiz et al. [38] who found that formalin related oxidative stress caused by this compound through production of reactive oxygen species (ROS), and depletion of the antioxidant enzymes such as superoxide dismutase and glutathione peroxidase and also caused destruction of mitochondria. These results confirmed by the histopathological alteration found in pancreatic tissue of animals treated alone or both with melamine and formaldehyde.

Disturbance in organic and inorganic constituents of serum in this work also might be owing to the damage caused by melamine and formaldehyde on the small and large intestine as previously obtained by Jeong et al. [36]. These findings confirmed by the histopathological findings as sloughing of villi into intestinal lumen, loss of villi, severe atrophy of villi and distortion of mucosal architecture of small intestine meanwhile, severe sloughing of intestinal mucosa and lymphocytic infiltration among mucosal propria and tunica submucosa and dysmorphism on large intestine which lead to malabsorption of digested element and so decrease their levels in blood.

CONCLUSION

In conclusion we found that both melamine and formaldehyde synergistic the toxic effect of each other on pancreatic tissue as they induced oxidative damage which lastly leading to maldigestion and malabsorption.

ACKNOWLEDGEMENT

Authors were greatly thankful to Dr/ Sameh M. Farouk, Lecturer of Histology, Faculty of Veterinary Medicine, Suez Canal University for his aid in reading the histopathological alterations.

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Cite this article

Mohamed WAM, El-Mandrawy SAM (2016) Functional and Cellular Exocrine Pancreatic Dysfunction in Male Mice Following Sub-Chronic Exposure to Melamine and Formaldehyde. *Ann Clin Pathol* 4(5): 1080.