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Hematobiochemical Changes During Subacute Toxicity of Melamine in Wistar Rats

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Abstract The present research work was conducted on 24 male and 24 female Wistar rats to study the hematobiochemical effects of repeated dose (28 days) of melamine. Wistar rats were randomly divided into 4 different groups with six males and six females in each group. The dose dependent reduction in body weight and feed consumption was observed in animals of group II, III and IV. The significant decrease in RBCs count, PCV, hemoglobin and MCV was recorded with increase in the dose of melamine in group IV. Moreover, significant decrease in MCH count was noticed in male and highly significant decrease in female from group IV animals whereas significant increase in total leucocyte count was noticed in group III and highly significant increase in group IV animals. The differential leucocyte count revealed significant increase in neutrophil count in group III and highly significant increase in group IV animals whereas significant decrease in lymphocyte count in animals of melamine treated group IV. No significant change in monocyte, eosinophil and basophil counts were observed in any treatment groups. AST, creatinine, BUN and uric acid values were significantly increased in treatment group III whereas highly significant increased in group IV. Moreover, ALT was increased significantly in group III and IV male animals. Whereas increased significantly and highly significantly in female animals of group III and IV respectively. The significant decrease in total protein and albumin was observed in treatment group III and highly significant decrease in group IV animals. The overall lesions gave impression that melamine was nephrotoxic as well as hepatotoxic in nature. The intensity and distribution of such lesions were more severe in rats of group IV, followed by rats of group III.

Keywords Wistar rats, Males, Female, Nephrotoxic, Hepatotoxic.

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Introduction

Melamine (tripolycyanamide) is an industrial chemical composed mainly of nitrogen (66%), carbon and hydrogen. Melamine is also a metabolite formed in the body of mammals that have ingested pesticide cyromazine. It has been reported that cyromazine can also be converted to melamine in plants Chan et al. [1]. In 2007, across the USA, cases of acute renal failure among cats and dogs were noticed by veterinarians and investigation revealed an epidemic of melamine contamination of pet food imported from

China. More than 1000 dogs and cats died in various countries due to renal failure caused by accumulated kidney stones WHO [2]. The occurrence of MEL exposure may possibly also be due to the use of triazine based pesticides (for instance cyromazine) used to control fly populations in cattle and poultry manures. When cyromazine (CYR) is dealkylized, one of the degradated products is MEL motivating the relevance of investigating the detection of MEL metabolized from CYR in poultry meat and eggs. Cyromazine is also used on field crops or sprayed onto fruits and vegetables Patakioutas et al. [3] detected MEL residues of less than 1 mg/kg on the edible parts of crops (tomato, lettuce and celery) after applying CYR. Melamine is also used as nitrogen source for slow release urea-based fertilizers and may be a major MEL contaminant in food and water Hilts and Pelletier [4]. In the preliminary investigation, the World Health Organization has provided a list of products that had tested positive for melamine from China included powdered infant formula, liquid milk, yogurt and powdered milk. Products reported for other countries included all listed product from China in addition to frozen dairy products, snack foods, frozen processed foods, ammonium bicarbonate, nondairy creamer, protein powder, dried egg powder, liquid eggs, whole eggs, and animal feed Gossner et al. [5]. WHO has set the tolerable daily intake (TDI) levels for humans at 0.2 mg/kg body weight Setiogi [6], and the maximum allowable MEL concentration in human foods at 2.5 mg/kg. The maximum allowable level for infant formula was set at 1 mg/kg. For animal feeds, the industry has also accepted 2.5 mg/kg as the maximum allowable MEL level. However, products containing excessive MEL residues could still surface in countries with no restriction policies.

Materials and Methods

The present study was designed to assess the sub acute toxicopathological effects of melamine in Wistar rats. The study was carried out at the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat. The parameters studied were clinical signs, mortality, body weight, feed intake, hematology, serum biochemistry and pathomorphology. The study was conducted on colony-bred 7-8 weeks old, adult male and female albino Wistar rats. Rats were procured from Division of Pharmacology and Toxicology, Cadila Pharmaceuticals Limited, Dholaka, Gujarat, India and were maintained under standard managemental conditions. The sub acute toxicopathological study of melamine was evaluated on 24 male and 24 female rats. All the 48 rats were randomly divided into 4 different groups. Each group consisted of 6 male and 6 female rats. The groups were numbered as group I to IV. The group I served as control and received only vehicle (Corn oil), while group II, III and IV received melamine at doses of 500 mg/kg (low dose), 1000 mg/kg (medium) and 2000 mg/kg body weight (high dose) respectively by oral gavage for 28 days of dosing period. At the end of 28^{th} day post treatment, blood was collected from retro-orbital plexus with the help of capillary tube as. Blood was collected in two aliquots. In one aliquot, K_3 EDTA (1-2 mg/ml) was added as anticoagulant and used for hematological estimation. In second aliquot, serum was separated from anticoagulant free blood for biochemical estimation. Moreover, urine was also collected and wet smears were prepared for microscopic examination.

Following hematological parameters were analyzed by automatic whole blood analyzer (MINDRAY BC-2800). (a) Total Erythrocyte Count (TEC), (b) Hemoglobin (Hb), (c) Packed Cell Volume (PCV), (d) MCV, MCH and MCHC, (e) Total Leukocyte Count (TLC), (f) Differential Leukocyte Count (DLC).

Serum was separated from anticoagulant free blood samples and refrigerated at -20°C for biochemical estimations. The following biochemical estimations were carried out by using automatic biochemical analyzer (Photometer BT 224) and diagnostic kits (Coral Limited ; Crest Biosystems). (a) Aspartate Aminotransferase (AST), (b) Alanine Aminotransferase (ALT), (c) Alkaline phosphatase (AKP), (d) Serum creatinine, (e) Blood urea nitrogen (BUN), (f) Uric acid (UA), (g) Total protein (TP), (h) Albumin, (i) Globulin (Calculated).

The data obtained from body weight, hematology parameters, biochemistry parameters and feed weights were subjected to statistical analysis. The statistical procedure used for analysis of above data

Table 1. Mean hematological values in different experimental groups. *Significant ($p < 0.05$), **Highly significant ($p < 0.01$).

Group No.	Dose (mg/kg)	Sex	RBC (X 10^6 / µL) Mean	SE	WBCs (X 10^3 / µL) Mean	SE	Differential WBCs Neutrophils (%) Mean	SE	Lymphocytes (%) Mean	SE
I	Control (0 mg/kg)	M	8.29	0.01	10.25	0.13	20.79	0.33	73.09	0.49
II	500 mg/kg	M	8.34	0.01	10.54	0.04	21.72	0.29	72.07	0.16
III	1000 mg/kg	M	8.24	0.03	10.66*	0.08	22.21*	0.35	70.82	0.26
IV	2000 mg/kg	M	8.19*	0.04	12.35**	0.20	23.47**	0.26	71.32*	0.20
I	Control (0 mg/kg)	F	8.10	0.17	9.25	0.80	22.62	1.71	71.72	1.69
II	500 mg/kg	F	7.54	0.11	9.81	0.89	23.55	1.33	70.79	1.20
III	1000 mg/kg	F	7.86	0.07	12.09*	0.84	27.06*	0.71	67.58	0.70
IV	2000 mg/kg	F	7.34*	0.23	13.28**	0.94	30.21**	1.36	65.37*	1.34

Table 1. Continued.

Group No.	Dose (mg/kg)	Sex	Differential WBCs Monocytes (%) Mean	SE	Eosinophils (%) Mean	SE	Basophils (%) Mean	SE
I	Control (0 mg/kg)	M	3.79	0.16	1.59	0.05	0.74	0.06
II	500 mg/kg	M	3.75	0.23	1.70	0.14	0.76	0.07
III	1000 mg/kg	M	3.48	0.18	1.79	0.25	0.69	0.06
IV	2000 mg/kg	M	3.34	0.14	1.31	0.12	0.56	0.07
I	Control (0 mg/kg)	F	3.65	0.15	1.38	0.10	0.63	0.05
II	500 mg/kg	F	3.35	0.47	1.56	0.19	0.75	0.08
III	1000 mg/kg	F	3.44	0.08	1.34	0.15	0.58	0.07
IV	2000 mg/kg	F	3.12	0.19	0.85	0.22	0.46	0.06

was unpaired two tailed student's *t* test where the $p < 0.05$ has been considered as statistically significant and $p < 0.01$ as highly significant. The obtained data in this study were described using the following statistical parameters viz. arithmetic mean (X), standard error of mean (SEM).

Results and Discussion

Hematological parameters of all the male and female animals of control group I (0 mg/kg), group II (500 mg/kg), group III (1000 mg/kg) and group IV (2000 mg/kg) were studied on 29th day of experiment (Tables 1 and 2). Hematological parameters studied for all the male and female animals were Red Blood Corpuscles (RBCs), Hemoglobin (Hb), Pack Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), White Blood Corpuscles (WBCs) and differential leucocyte counts (DLC). The hematological values obtained in the control rats were well within the normal range. There was no significant ($p < 0.05$) effect on the RBCs count in melamine treated rats of group II and III as compared to control whereas gradual decrease in RBCs count observed in the treatment group IV. The mean values of Hb and PCV also decreased significantly in treatment group IV in male and female rats. Significant decrease in mean MCV and highly significant decrease in MCH in experimental group IV was observed while group II

Table 2. Mean hematological values in different experimental groups. *Significant ($p < 0.05$), **Highly significant ($p < 0.01$).

Group No.	Dose (mg/kg)	Sex	Hb (g/dL)		PCV (%)		MCV (fL)		MCH (pg)		MCHC g/dL)	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
I	Control (0 mg/kg)	M	15.37	0.02	45.13	0.06	58.52	0.04	19.12	0.30	33.40	0.22
II	500 mg/kg	M	15.41	0.01	45.43	0.13	58.55	0.05	18.98	0.18	33.62	0.39
III	1000 mg/kg	M	15.24	0.06	44.83	0.12	58.55	0.05	18.67	0.40	33.62	0.17
IV	2000 mg/kg	M	15.12*	0.08	43.64*	0.39	56.11*	0.54	17.80*	0.45	34.00	0.40
I	Control (0 mg/kg)	F	15.38	0.34	44.82	0.05	58.43	0.04	19.00	0.17	33.83	0.23
II	500 mg/kg	F	14.52	0.34	45.09	0.34	59.09	0.92	18.72	0.26	33.72	0.41
III	1000 mg/kg	F	15.37	0.29	44.49	0.43	58.55	0.95	18.36	0.17	34.40	0.11
IV	2000 mg/kg	F	13.42*	0.49	43.27*	0.40	57.25	0.33	17.44**	0.18	34.75	0.44

and III did not differ significantly ($p < 0.05$). Hematological picture revealed microcytic hypochromic anemia in high dose group IV due to chronic blood loss.

The reason for anemia in melamine toxicity might be due to kidney damage affecting erythropoietin secretion. Moreover, corrosive and irritant effect of MEL had caused hemorrhage in the urinary bladder leading to hematuria and anemia. Another adding factor that, ulcers in the digestive tract might have resulted in gradual blood loss. Wang et al. [7], further suggested that MEL-cyanurate complex damages the membrane of erythrocytes leading to hemolysis. So, hemolysis might be another possible cause of anemia. In the present, study male and female animals in high dose group showed hemosiderosis in spleen indicating hemolysis. Decrease feed consumption leading to hypoproteinemia could be another possible reason for lower hemoglobin seen in the present study. Similar observations on the values of MCV and MCH were made by Chen et al. [8] in their study. They reported MCV and MCH values significantly declined in groups 4 and 5 after feeding rats with levels of 50%–100% (w/w) of MEL contaminated pet food for three months. In present study, the mean values of TLC were increased highly significant in high dose group rats with neutrophillia and lymphopenia. An increased leucocyte count due to in-

Table 3. Mean values of serum biochemical parameters in males of different experimental groups. *Significant ($p < 0.05$), **Highly significant ($p < 0.01$).

Biochemical parameter	Group I Control (0 mg/kg)		Group II 500 mg/kg		Group III 1000 mg/kg		Group IV 2000 mg/kg	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
AST (IU/L)	153.40	2.86	149.38	2.55	170.27*	5.65	184.35**	6.24
ALT (IU/L)	41.32	2.36	43.42	2.01	48.93*	1.02	49.25*	1.20
AKP (IU/L)	158.45	3.44	159.72	1.39	162.71	3.39	164.28	3.75
Creatinine (mg/dL)	0.70	0.07	0.74	0.05	1.15*	0.18	2.13**	0.10
BUN (mg/dL)	23.17	0.65	24.82	0.87	28.33*	0.91	38.55**	1.07
Uric acid (mg/dL)	1.632	0.15	1.687	0.13	2.307*	0.21	2.517**	0.23
Total protein (g/dL)	7.04	0.23	6.87	0.14	6.25*	0.23	5.51**	0.16
Albumin (g/dL)	4.00	0.05	3.84	0.07	3.71*	0.10	3.14**	0.06
Globulin (g/dL)	3.04	0.27	3.00	0.16	2.54	0.16	2.37	0.19

Table 4. Mean values of serum biochemical parameters in females of different experimental groups. *Significant ($p < 0.05$), **Highly significant ($p < 0.01$).

Biochemical parameter	Group I Control (0 mg/kg) Mean	SE	Group II 500 mg/kg Mean	SE	Group III 1000 mg/kg Mean	SE	Group IV 2000 mg/kg Mean	SE
AST (IU/L)	152.55	2.31	150.93	1.32	165.27*	3.59	182.03**	4.78
ALT (IU/L)	38.20	2.29	39.08	1.50	44.47*	0.69	48.20**	1.90
AKP (IU/L)	152.99	1.03	151.83	1.28	153,91	2.16	155.50	6.74
Creatinine (mg/dL)	0.72	0.05	0.76	0.07	1.24*	0.18	2.01**	0.20
BUN (mg/dL)	23.45	0.90	25.39	1.05	27.14*	0.96	38.37**	3.01
Uric acid (mg/dL)	1.197	0.22	1.44	0.24	2.038*	0.29	2.685**	0.27
Total protein (g/dL)	7.19	0.09	6.84	0.20	6.56*	0.19	6.04**	0.18
Albumin (g/dL)	4.18	0.07	4.02	0.05	3.83*	0.12	3.32**	0.05
Globulin (g/dL)	3.01	0.12	2.82	0.17	2.73	0.20	2.72	0.22

crease in mature neutrophils that were likely related to the inflammatory events that occurred in the kidney and liver. Likewise, a secondary inflammation induced by the rupture of tubules obstructed by MEL crystals might be the reason for elevation in TLC and neutrophils. The decreased lymphocyte count might be related to stress induced by toxicity. The present finding of increase in WBCs count was in agreement with the previous observations of Chen et al. [8] during their study on rats. They fed rats with levels of 10% to 100% (w/w) of contaminated pet food for three months and observed that rats fed with 10%, 20%, and 50% diets experienced significantly elevated white blood cell counts, mainly, segmented neutrophils. Likewise, Choi et al. [9] evaluated comparative nephrotoxicity induced by MEL, CYA or a mixture of both chemicals in either sprague-dawley rats or renal cell lines. Male sprague-dawley rats were administered MEL, CA, and a mixture of both melamine and cyanuric acid (MC) at 5 doses each with 10-fold dose interval daily by oral gavage for 7 days as follows: MEL at 0.0315, 0.315, 3.15, 31.5, and 315 mg/kg ; CA at 0.025, 0.25, 2.5, 25, and 250 mg/kg, and MC: [1×: (0.0315 + 0.025), 10×: (0.315+ 0.25), 100×: (3.15 + 2.5), 1000×: (31.5+25), and (315+250) mg/kg]. They found that the number of white blood cells (WBC) was significantly elevated in a dose-dependent manner in the 1000×MC group compared with control. In present study, increase in the neutrophil count was observed with decrease in lymphocyte count in high dose group. Similar observation was also made by Chen et al. [8]. Moreover, Choi et al. [9] showed that lymphocytes were significantly reduced in a dose-dependent manner in the 1000×MC group compared with control during rat study. No significant changes in monocyte, eosinophil and basophil counts were observed in any treatment groups compared to control group.

Biochemical parameters of all the male and female animals of group I (control group), group II (500 mg/kg), group III (1000 mg/kg) and group IV (2000 mg/kg) were studied on 29th day of experiment. Biochemical parameters studied for the entire male and female animals were Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Creatinine, Blood urea nitrogen (BUN), Uric acid (UA), Total protein (TP), Albumin and Globulin.

In the present study, mid dose of melamine i.e. 1000 mg/kg b. wt/day caused significant increase in the value of AST. The highest dose of melamine i.e. 2000 mg/kg b. wt/day caused highly significant increase in the value of AST. These findings are in accordance with the findings of Brand [10]. They reported elevated values for serum AST linearly with increasing levels of melamine in the diet of Ross broiler chicks fed MEL at 0 to 3.0% for 21 days. In present study, elevated level of AST suggested damage caused by melamine toxicity to the liver. In the present study, mid dose of melamine i.e. 1000 mg/kg b. wt/day caused significant increase in the value of ALT. The highest dose of melamine i.e. 2000 mg/kg b. wt/day caused highly significant increase in the value of ALT. ALT enzyme is liver specific and present in hepatocyte cytoplasm. Increased ALT level in present

study might be due to liver damage in the rats exposed to 1000 mg/kg b. wt/day and 2000 mg/kg b. wt/day administration of melamine. In the present study, no significant change observed in the mean values of AKP of melamine treated groups of rats. Though the MEL crystals were found in the bile duct and gall bladder, probably they are not causing bile stasis. Elevated levels of AST and ALT suggest hepatocellular damage. These results indicate that the reduction in renal filtration rate might be due to toxic effect of melamine on kidney. The histopathological observation on kidney tissue also revealed crystals in the tubules leads to tubular degeneration and necrosis in melamine treated groups. These increased values are indicative of kidney damage which is reflected in terms of an increase in serum creatinine levels. These findings are in agreement with the findings of previous study by Choi et al. [9]. They observed that serum creatinine levels, markedly increased in a dose-dependent manner in the high-dose MC groups when rats were fed mixture of both melamine and cyanuric acid (MC) at 5 doses each with 10-fold dose interval daily by oral gavage for 7 days. Dobson et al. [11] who conducted study on rat reported an increased creatinine clearance with low urine pH for both MEL + CYA mixtures. Moreover Chen et al. [8] observed significantly elevated serum creatinine value after feeding rats the diet of 50%–100% for three months. Similar findings were also noted by Jacob et al. [12] and Wang et al. [7] during their study on rat. Moreover, similar observations were also made by Brown et al. [13] in dogs and cats. In present study, there was highly significant increase in the levels of BUN in melamine treated group IV and significant increase in group III. However, the group receiving 500 mg/kg b. wt/day showed slight increase in BUN but not at significant level. These values were supported by histopathological alteration, where there was dose dependant increase in severity of the cell injury in tubular epithelium of kidney. This indicated that the significant alterations noted in the BUN levels could be attributed to reduced kidney function caused by tubular damage. The present findings are in accordance with previous reports by Dobson et al. [11] who observed the increase in the BUN value substantially higher than normal in rats fed with triazine mixture containing MEL (400 mg/kg), ammeline ammelide and cyanuric acid (40 mg/kg each) in the feed. Chen et al. [8] also showed that significant elevated serum BUN in rats fed with the diet of 50%–100% for three months. Similarly, Choi et al. [9] reported blood urea nitrogen (BUN) and serum creatinine (CREA) levels in rats, markedly increased in a dose-dependent manner in MC groups. Likewise, dose dependent elevation in BUN value was reported by Jacob et al. [12], and Wang et al. [7] during their experiments on rats. Similarly, increased level of BUN was also reported by Brown et al. [13] in dogs and cats. In present study the data showed that serum concentration of UA was increased by MEL exposure, suggesting that kidney function was affected in the animals, especially in the group III and IV. These findings of the present study are in accordance with the findings of previous study by Dobson et al. [11] who observed the increase in the UA value substantially higher than normal in rats fed with triazine mixture containing MEL (400 mg/kg), ammeline, ammelide and cyanuric acid (40 mg/kg each) in the feed. Likewise, Wang et al. [7] noted the concentration of uric acid in dams was significantly increased when MEL was respectively administered at 0, 40 and 400 mg/kg body weight by daily gavage from gestation day 13 to 20 to control, low and high melamine groups of pregnant female F344 rats. Moreover, Brand et al. [10] who showed that values for serum UA increased linearly with increasing levels of MEL in the diet of broiler chickens.

Brand [10] observed reduction in levels of serum albumin and total protein in birds fed 3% MEL. Similar findings of decrease albumin were also reported by Chen et al. [8] in rats exposed to MEL containing diet of 50%–100% for three months (Tables 3 and 4). Globulin has no significant change in male and female of all the melamine treated groups. There was significant dose dependent reduction in the total protein and albumin. This could be correlated with nephrotoxicity induced by MEL.

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