Asian Journal of Biochemistry

ISSN 1815-9923

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Biochemistry 10 (3): 125-131, 2015
ISSN 1815-9923 / DOI: 10.3923/ajb.2015.125.131

Effect of Melamine Adulteration on Body Weight, Liver and Kidney Histology and Liver Function Indices of Albino Rats

S. Shehu, R.S.U. Wasagu, M. Lawal and A.K. Albert
Department of Biochemistry, Faculty of Science, Usmanu Danfodiyo University, Sokoto, Nigeria

Corresponding Author: S. Shehu, Department of Biochemistry, Faculty of Science, Usmanu Danfodiyo University, Sokoto, Nigeria

ABSTRACT

The effect of melamine adulteration on body weight, liver and kidney histology and liver function using feed containing 0, 4, 8, 12 and 16 mg of melamine per kg diet administered *ad labium* to wistar rats were studied. Kidney histology was affected at concentrations ≥8 mg kg^{-1} by the onset of mild interstitial chronic inflammation while the liver histology of all test animals appeared normal. Results of body weight change showed significant ($p<0.05$) increase at the highest dosed group though, body weight increase was observed in all animals. The subchronic toxicity study on liver function parameters showed significant ($p<0.05$) increase in ALT activity at all doses while, total protein concentration was significantly affected ($p<0.05$) in the highest dosed group. Melamine should therefore, be absent from feed especially at concentrations approaching milligram quantity in order to safe guard health.

Key words: Melamine, kidney histology, liver histology and liver function

INTRODUCTION

Melamine (MEL) became an adulterant of much discussion after it was associated with the deaths of pets in America and six children in China in 2007 and 2008, respectively (Baynes *et al*., 2008). Sequel to this, the National Agency for Food and Drug Administration and Control (NAFDAC) in Nigeria and Food Agencies of other countries in line with the regulations from World Health organization (WHO), Food and Agricultural Organization (FAO) and International Food Safety Authorities Network has subjected both internationally and locally produced food items of milk and milk derived ingredients to routine melamine detection and quantification tests (Gossner *et al*., 2009).

Hau *et al*. (2009) reported that addition of 1 g of melamine to 1 L of milk falsely increases the protein content by 0.4% and when 3.1 g of melamine is dissolved in at room temperature; protein content is falsely increased by 1.2%, which roughly leads to an overestimation of protein content in liquid milk by 30% (Hau *et al*., 2009).

Melamine, an organic base and trimer of cyanamide with a 1, 3, 5-triazine skeleton (Fig. 1), contains 66% nitrogen by weight (Chan *et al*., 2008) and has the following structure shown in Fig. 1.

Melamine was first synthesized by the German Chemist Justus von Liebig in 1834 by first converting calcium cyanamide into dicyandiamide which he then heated above its melting temperature to produce MEL.

Today, most industrial manufacturers use urea in the following reaction to produce MEL:

$$6\ (NH_2)_2CO \rightarrow C_3H_6N_6 + 6\ NH_3 + 3\ CO_2$$ (Bann and Miller, 1958)

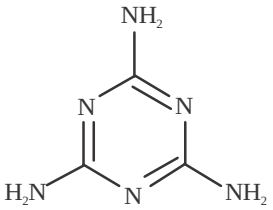

Fig. 1: Structure of melamine

Mast *et al*. (1983) as cited in Hau *et al*. (2009) reported that MEL is rapidly absorbed and attains maximal plasma concentrations in 60 min following administration of a single oral dose to male Fischer 344 rats and appears to distribute to the body water, as concentrations in blood, plasma and liver are similar.

China's General Administration of Quality Supervision, Inspection and Quarantine gave results of an investigation into the extent of MEL contamination of dairy products (Chan *et al*., 2008; WHO, 2008a, b, 2009) stating that of the 109 manufacturers of domestic powdered infant formula whose products were tested, 69 batches produced by 22 companies contained detectable levels of MEL. It is believed that melamine was added to raw ingredients to increase their apparent protein content, after these products had been diluted with water (Hau *et al*., 2009).

Radio labelled MEL measurements supported the conclusion that urinary elimination is virtually the only route of excretion (93±4% of dose) while, excretion of MEL by respiration and faeces were each less than 1% of the administered dosage (Mast *et al*., 1983).

Melamine was reported by Worzalla *et al*. (1974) and Mast *et al*. (1983) cited in WHO expert meeting report (WHO, 2008a), to be eliminated essentially unchanged by the kidney and that the percentage of urinary ^{14}C present as MEL was the same as the radiochemical purity of the dosed material since, almost all (97-100%) ^{14}C in the liver, blood and plasma was present as MEL, leading to the conclusion that the male Fischer 344 rats do not metabolize MEL.

To evaluate the *in vivo* toxic effects of melamine ingested as adulterant in feed and establish safe concentration limits, above which liver and kidney toxicity is experienced and body weight is negatively affected.

MATERIALS AND METHODS

Melamine: Two hundred and fifty grams of MEL (1, 3, 5-triazine-2, 4, 6-triamine) was purchased from Steve Moore chemical company Ltd, Zaria, Nigeria.

Experimental animals: Twenty Wistar strain albino rats weighing an average of 100 g were obtained from the Pharmacy Department of Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto. They were allowed to acclimatize to the laboratory environment for 7 days in the animal house of Biochemistry Department, Usmanu Danfodiyo University, Sokoto.

Sub-chronic toxicity test: Repeated toxicity study was carried out according to the OECD guideline (OECD., 1998). The animals were divided into five groups of four rats each. Groups 2, 3, 4 and 5 were placed on fresh clean water and a standard feed containing 4, 8, 12 and 16 mg of melamine as adulterant/kg diet, for 28 days. Group 1 served as control, receiving feed containing 0 mg of MEL. The body weights of the animals were evaluated a day before the start of administration and then weekly for four weeks.

Collection of blood samples: On the 29th day, the animals were sacrificed after overnight fast and the blood samples were collected into labeled centrifuge tubes and allowed to stand for 10 min before being subjected to centrifugation at 4000 rpm for 15 min. The serum was separated from the whole blood using Pasteur pipette and transferred to labeled dry clean serum tubes and then kept refrigerated.

Liver function tests: Total protein was determined by Biuret Method (Hiller, 1926), based on reaction of cupric ions in an alkaline medium, which interacts with protein peptide bonds resulting in the formation of a blue-violet complex (the so-called biuret reaction). Albumin was determined by Bromocresol Green method, measured based on its quantitative binding to the indicator 3, 3, 5, 5-tetrabromo cresol sulphonephthalein (Bromocresol green, BCG). Globulin was determined by subtracting albumin from the total protein concentration. Aspartate Aminotransferase (AST) was determined by monitoring the concentration of oxaloacetate hydrazone formed from 2, 4-dinitrophenylhydrazine (Reitman and Frankel, 1957). Alanine Aminotransferase (ALT) was determined by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine (Reitman and Frankel, 1957). Alkaline Phosphatase (ALP) was measured based on ρ-nitrophenyl phosphate which is hydrolyzed to ρ-nitrophenol and inorganic phosphate by alkaline phosphatase at pH 10.4 (Kind and King, 1954).

Histopathological analysis

Tissue collection: The tissues/organs of interest were removed from the body of the test animals following their anesthetization with chloroform and then fixed in 10% formol saline to prevent decay prior to the histopathological examination.

Gross examination: Tissues removed from the body of the test animals were taken to the Histopathology Department of the Usmanu Danfodiyo University Teaching Hospital for examination by a histopathologist. Gross examination was performed to describe the specimens and placed into a small plastic cassette which holds the tissue while it is being processed to a paraffin block.

Tissue processing: The tissues were processed into thin microscopic sections of 6-8 μm, by paraffin fixation. Firstly, the tissues were dehydrated with an alcohol in increasing concentrations; 70, 90 and 100%. The cassette containing the tissues allows the reagents to get in contact freely with the tissues. This was followed by 'clearing' of dehydrant with xylene (a substance that is miscible with the embedding medium, paraffin). The tissues were then impregnated with wax (paraffin) at a temperature of about 54-60°C for a period of about 4 h, to remove the clearing agent and moisten the tissues. Finally, the impregnated tissues were placed in a mold with their labels and then fresh melted paraffin wax was poured onto it and allowed to settle and solidify. Once the block had cooled sufficiently to form a surface skin, it was immersed in cold water to cool it rapidly. After the block has completely cooled, it was cut into individual blocks. This 'blocking' allowed the tissues to be cut into small sections using a microtome for subsequent microscopic examination.

Staining and microscopy: In order to view the tissues under a microscope, the sections were stained with dyes, hematoxylin and eosin (H and E pigments). Hematoxylin stains the nuclei blue, while, eosin stains the cytoplasm and the extracellular connective tissue matrix pink. This revealed

the cellular components of the tissues to be examined. Prior to staining, the sections were first deparaffinized by running them through xylene. The histological slides were examined under a microscope.

Statistics: All data expressed as Mean±Standard Deviation (n = 4) was analyzed by one way analysis of variance (ANOVA) using graph pad INSTAT3 software (San Diego, USA) and considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Tissue enzymes indicate cellular damage long before structural damage of tissues can be determined by conventional histological technique. The liver being the primary organ for the detoxification and destruction of drugs and other chemicals and the kidney, being the major excretory organ could be assessed to establish the safety of a substance (Gupta *et al*., 2004). Histopathological appearance (Fig. 2) of the liver of all test animals that ingested MEL tainted feed ranging from 0-16 mg kg^{-1} diet for 28 days show normal hepatic architecture and portal triad; a component of the hepatic lobule, which consists of a hepatic artery, common bile duct, hepatic portal vein and lymphatic vessels. Kidneys of group 1 and 2 test animals fed with MEL at adulterant concentration of 0 and 4 mg kg^{-1} (Fig. 3) show normal histological appearance consisting of normal renal tubules, glomerulus and blood vessels while, those given MEL doses ≥8 mg kg^{-1} diet showed mildly increasing interstitial infiltrations by lymphocytes, a symptom often consistent with interstitial chronic inflammation. Progressive increase in body weight (Table 1) was seen in all rats over the 28 days test period with the highest increment seen in the control group and the least in the highly dosed groups 4 and 5 administered 12 and 16 mg of MEL per kg of diet.

Serum ALP, ALT and AST are useful indices for identifying inflammation, necrosis and determining liver function (Tilkian *et al*., 1979) although ALT measurements are more liver specific

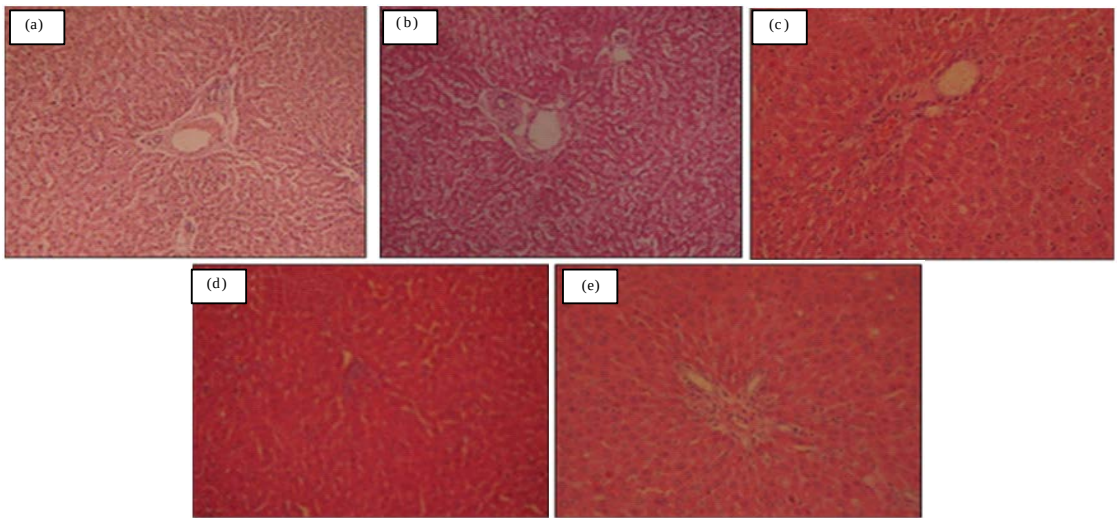

Fig. 2(a-e): Effect of 28 days administration of feed adulterated with varying doses of melamine on the liver histology of experimental rats, (a) 0 mg kg^{-1}, (b) 4 mg kg^{-1}, (c) 8 mg kg^{-1}, (d) 12 mg kg^{-1} and (e) 16 mg kg^{-1}. Normal histological appearance consisting of hexagonally patterned hepatocytes with the portal triad and central vein appeared in the liver sections of the rats

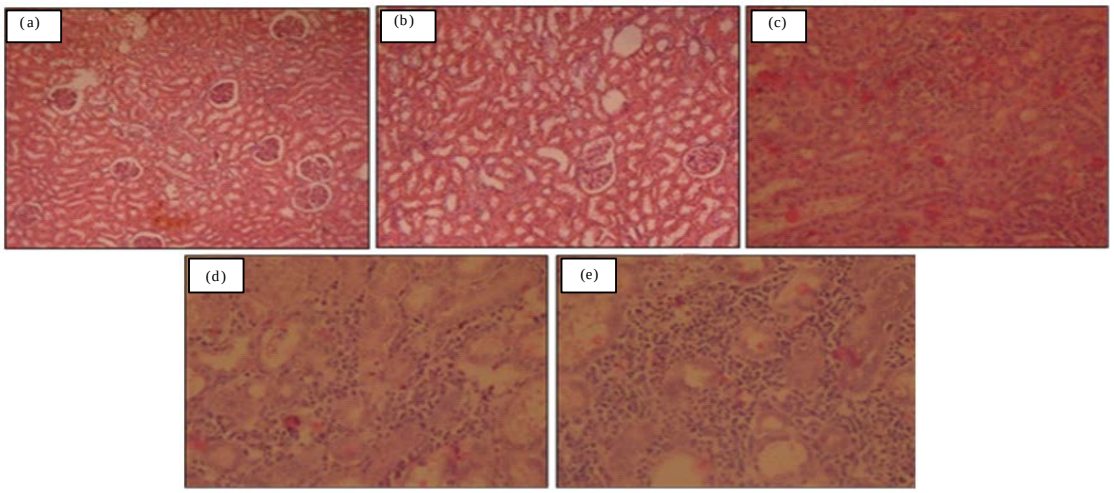

Fig. 3(a-e): Effect of 28 days administration of feed adulterated with varying doses of melamine on the kidney histology of experimental rats. Histological appearance of kidney sections (a, b) Normal histological appearance consisting of normal tubules, glomeruli and interstitial blood vessels, (c) Mild interstitial infiltration by lymphocytes and (d, e) Interstitial infiltration by lymphocytes, consistent with interstitial chronic inflammation

Table 1: Effect of administration of feed adulterated with melamine on the body weight of experimental rats after four weeks

		Weeks				
Groups	Dose (mg kg^{-1})	0	1	2	3	4
1 (Control)	0	75.50±9.678	90.00±9.626*	106.00±16.833*	121.50±17.098*	135.75±14.569*
2	4	130.25±16.174	138.25±14.080**	147.50±16.583**	156.50±22.290**	166.00±24.29**
3	8	123.25±15.218	128.75±15.521**	136.75±12.894**	147.50±8.386**	156.00±5.715*
4	12	106.50±19.296	109.25±22.277**	110.75±19.738**	112.75±19.050**	116.25±17.115**
5	16	79.50±4.203	83.50±4.203*	88.00±3.651*	91.50±3.000*	95.50±3.697*

Values are Mean±SD, n = 4, *Significantly different from control (p<0.05), **Not significantly different from control (p<0.05)

Table 2: Serum liver markers after 28 days of administration of melamine adulterated feed

Groups	Dose (mg kg^{-1})	ALT (U L^{-1})	AST (U L^{-1})	ALP (U L^{-1})	TP (g dL^{-1})	ALB (g dL^{-1})	GLB (g dL^{-1})
1	0	23.74±5.942	42.80±10.844	24.04±2.217	9.63±0.751	5.55±0.875	4.09±0.444
2	4	38.75±6.821*	50.85±12.613**	26.30±4.541**	9.27±0.933**	5.37±1.091**	3.90±0.635**
3	8	54.75±5.625*	56.04±12.923**	27.73±6.726**	8.69±0.614**	4.90±0.927**	3.79±0.380**
4	12	61.02±5.857*	62.55±10.386**	28.45±6.050**	8.32±0.541**	4.85±0.786**	3.47±0.368**
5	16	64.95±6.581*	61.57±13.779**	29.36±4.655**	7.57±0.569*	4.21±0.327**	3.35±0.349**

Values are Mean±SD. *Significantly different from control (p<0.05), **Not significantly different from control (p<0.05), n = 4. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, TP: Total protein, ALB: Albumin and GLB: Globulin

than the AST and its activity is usually greater than AST activity at early or acute hepatocellular disease (Table 2). Aspartate aminotransferase (AST) on the other hand, tend to be released more than ALT in chronic liver diseases like cirrhosis (Whitby *et al*., 1989). Alkaline phosphatase (ALP) is a useful diagnostic screening and follows up tool for cholestatic hepatobiliary lesions (Wolf, 1978), because cholestasis is the main if not the only liver disease responsible for increased plasma ALP activity. Thus, a normal ALP activity in the presence of abnormal levels of other liver function parameters may suggest liver pathology other than obstruction (Tilkian *et al*., 1979). Test for serum markers showed gradual increase in AST, a more heightened increase in AST and the most significant (p<0.05) increase were seen in the ALT result with increase in MEL adulterant concentration.

Decreasing total protein concentration observed may be due to malnutrition, liver and/or kidney disorder or a disorder in which protein is not digested or absorbed properly and is hence, excreted in urine or low albumin concentration in instances of liver disease, acute infection, malabsorption and nephritic syndrome since, albumin is made in the liver. The serum globulin concentration calculated by subtracting albumin concentration from total protein concentration also showed steady ($p<0.05$) decreases with increased concentration of MEL adulterant.

CONCLUSION

Melamine (MEL) as an adulterant is nephrotoxic with reference to histological architecture observed in the kidneys of test animals at concentrations above 8 mg kg^{-1}, however, this need to be supported with other biochemical parameters, such as urea and creatinine levels. Mild hepatotoxicity was evident from the elevated levels of serum marker enzymes (ALT, AST and ALP) and decreasing total protein, albumin and globulin concentration even though, the deteriorating state of the liver and the gradual loss of its function was not detected by conventional histological study.

REFERENCES

Bann, B. and S.A. Miller, 1958. Melamine and derivatives of melamine. Chem. Rev., 58: 131-172.

Baynes, E.R., G. Smith, E.S. Mason, E. Barrett, E.B. Barlow and E.J. Riviere, 2008. Pharmacokinetics of melamine in pigs following intravenous administration. Food Chem. Toxicol., 46: 1196-1200.

Chan, E.Y.Y., S.M. Griffiths and C.W. Chan, 2008. Public-health risks of melamine in milk products. The Lancet, 372: 1444-1445.

Gossner, C.M.E., J. Schlundt, P.B. Embarek, S. Hird, D. Lo-Fo-Wong and J.J.O. Beltran *et al*., 2009. The melamine incident: Implications for international food and feed safety. Environ. Health Perspect., 117: 1803-1808.

Gupta, M., U.K. Mazumder, T.S. Kumar, P. Gomathi and R.S. Kumar, 2004. Antioxidant and hepatoprotective effects of *Buhinia racemosa* against paracetamol and carbon tetrachloride induced liver damage in rats. Iran. J. Pharmacol. Therapeut., 3: 12-20.

Hau, A.K.C., T.H. Kwan and P.K.T. Li, 2009. Melamine toxicity and the kidney. J. Am. Soc. Nephrol., 20: 245-250.

Hiller, A., 1926. Biuret method. Proc. Soc. Exp. Biol. Med., 24: 385-390.

Kind, P.R. and E.J. King, 1954. Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. J. Clin. Pathol., 7: 322-326.

Mast, R.W., A.R. Jeffcoat, B.M. Sadler, R.C. Kraska and M.A. Friedman, 1983. Metabolism, disposition and excretion of [^{14}C]melamine in male Fischer 344 rats. Food Chem. Toxicol., 21: 807-810.

OECD., 1998. Screening Information Dataset (SIDS) for melamine. CAS No. 108-78-1, Organisation for Economic Co-operation and Development, Paris.

Reitman, S. and S. Frankel, 1957. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol., 28: 56-63.

Tilkian, S.M., M.B. Conover and A.G. Tilkian, 1979. Clinical Implications of Laboratory Tests. C.VC.Mosby Co., St Louis, Toronto, London, pp: 3-44.

WHO., 2008a. Melamine-contaminated powdered infant formula, China. Weekly Epidemiological Record, No. 39, World Health Organization, Geneva, pp: 349-356.

WHO., 2008b. Melamine-contamination event, China. World Health Organization, Geneva, September-October 2008.

WHO., 2009. Expert meeting to review toxicological and health aspect of melamine and cyanuric acid. World Health Organization, Geneva, December 1-4, 2008.

Whitby, L.G., A.F. Smith and G.J. Backet, 1989. Lecture Notes on Clinical Chemistry. 4th Edn., Blackwell Scientific Publications, Oxford, London Edinburg, pp: 138-178.

Wolf, P.L., 1978. Clinical significance of an increased or decreased serum alkaline phosphatase level. Arch. Pathol. Lab. Med., 102: 497-501.

Worzalla, J.F., B.D. Kaiman, B.M. Johnson, G. Ramirez and G.T. Bryan, 1974. Metabolism of hexamethylmelamine-ring-^{14}C in rats and man. Cancer Res., 34: 2669-2674.