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Original Scientific Article

MELAMINE-INDUCED NEPHROTOXICITY IN WEANED PIGLETS IN SERBIA

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Summary: Melamine is not approved for use in animal feed, although evidence of melamine poisoning in pigs has been found. Melamine in animal feed can induce nephrolithiasis, chronic kidney inflammation, bladder carcinoma and even death in animals. In September 2011, nine hundred 40–60-day-old piglets from a commercial finishing farm in Serbia developed anorexia, wasting, polydipsia and lethargy. At necropsy, the main macroscopic finding in ten necropsied piglets was observed on the kidneys. The kidneys were slightly enlarged and firm in consistency. The cortical surface was wrinkled, with a diffuse distribution of the yellow foci in the cortex and medulla. Microscopically, these yellow foci were accumulations of crystals located predominantly within the lumina of the dilated distal straight and convoluted tubules and collecting ducts. The crystals appeared variable and birefringent when viewed under polarized light. Two types of crystals were observed: the first type had several dark striations radiating from the eosinophilic round centre, while the second type consisted of pale green to brown irregular striated aggregates without an eosinophilic centre. In all cases, multifocally, there were moderate aggregates of lymphocytes, plasma cells, macrophages and multinucleated giant cells within the interstitium.

From the clinical signs, necropsy findings, histochemical and toxicological results, it was concluded that melamine-contaminated feed was the primary cause of nephrotoxicity and death in the observed piglets. To our knowledge, this is the first report of melamine poisoning in pigs in Serbia.

Key words: melamine; kidney; weaned piglets; nephrotoxicity

Introduction

Melamine (1,3,5-triazine-2,4,6-triamine) is a chemical product that used to be sporadically mixed into animal feeds to boost protein content; it gives a false elevation of the total protein content because the added melamine nitrogen is non-protein nitrogen. Melamine has no nutritive value and cannot be a substitute for proteins. Usually, melamine is used in the production of melamine resins, plastics, glues and inks (1, 2). Alkaline hydrolysis of melamine can produce three melamine derivatives: ammeline, ammelide, and cyanuric acid. Separately, melamine and cyanuric acid are relatively nontoxic in mammals (3). However, Brand (4) found that the addition of cyanuric acid to a diet containing melamine caused less toxicity in poultry than a diet contaminated with only melamine. Melamine and melamine derivatives cause poisoning in animals and humans (1, 3, 5-7). Melamine in animal feed can induce nephrolithiasis, chronic kidney inflammation, bladder carcinoma and even death in animals (1).

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Recent cases of nephrolithiasis and acute kidney injury among children in China have been linked to ingestion of milk-based infant formula contaminated with melamine. By November 2008, an estimated 300,000 victims had been reported, with six infants dying from kidney stones and other kidney damage, and a further 50,000 babies hospitalized (1, 7, 8). Although used mainly by the chemical industry, melamine was recently implicated in a foodborne outbreak of renal disease in domestic cats and dogs in the United States and Korea. In those cases, commercial pet food was adulterated with melamine (8-11). In swine, poisoning with melamine is characterized by anorexia, wasting, polydipsia, slight pallor and sometimes jaundice, lethargy and increased mortality. Sometimes there are no clinical symptoms (3, 5, 6). In one investigation, fish and pigs were fed a target dosage of melamine (400 mg/kg b.m.), cyanuric acid (400 mg/kg b.m.), or melamine and cyanuric acid (400 mg of each compound/kg b.m.) daily for three days. All animals fed the combination of melamine and cyanuric acid developed goldbrown renal crystals arranged in radial spheres (spherulites). Spectral analyses of crystals were consistent with melamine-cyanurate complex crystals. Although melamine and cyanuric acid appeared to have low toxicity when administered separately, they induced extensive renal crystal formation when administered concurrently (12). In one investigation, crystals were also found in one of the two pigs fed only melamine (200 mg/kg body mass per day) (13).

Melamine is not metabolized and is rapidly eliminated in the urine. One study, in which pigs were inoculated with melamine intravenously, demonstrated that melamine is rapidly eliminated by the kidney and probably not widely distributed to various tissues in the pig, and there should be no concerns about extensive binding to tissues that may be consumed by the public (14). However, methods for fast and on-site screening of melamine residue in animal tissues in order to eliminate the potential threat to human health have been developed (2).

In this paper, a case of accidental melamine poisoning of weaned piglets is described.

Material and Methods

Animals

In September 2011, nine hundred 40–60-day old piglets from a commercial finishing farm with 1500 sows, developed anorexia, wasting, polydipsia and lethargy. The piglets were fed with a complete feed mixture for weaned pigs. Feed intake gradually decreased by 20% to 40%. The morbidity rate gradually rose from 25% to 40% over a period of three weeks. Mortality began as early as three weeks after weaning and was 12% of the population of post-weaning piglets.

Pathological Examination

Necropsy was performed on the carcasses of ten clinically diseased weaned piglets, six males and four females, between 50 and 60 days old. For histopathological examination, samples of kidney tissue were taken, fixed in a 10% buffered formalin, routinely processed and embedded in paraffin blocks. Paraffin sections 4 μm thick were stained with haematoxylin and eosin (HE), Masson's trichrome and Periodic Acid Schiff (PAS), according to standard staining protocols, and the 72-hour Oil Red O (ORO72h) method described by Thompson et al. (10). All slides were viewed with light microscope Olympus BX51, and photos were taken with an Olympus Color View III camera. For the analysis of the observed crystals, slides were also viewed under polarized light.

Toxicological analysis

A milk replacer that was mixed in the feed for weaned piglets was suspected to be the source of the melamine; therefore, a sample was taken for toxicological analysis. The melamine content in pig milk replacer sample was determined with the HPLC/UV method. Extraction was carried out with trichloracetic acid and acetonitrile, followed by purification using cation-exchange SPE columns (Phenomenex Strata X-C). Melamine was eluted from the columns using 5% ammonium hydroxide in methanol. The eluate was evaporated to dryness in a nitrogen stream, and the dry residue was reconstituted in 1 mL of mobile phase, filtered through 0.22 μm nylon syringe filters, and 20 μl of extract was injected into HPLC system.

Chromatographic analysis was performed with a Waters Alliance 2695 Separation Module (Waters, Milford, USA) and UV-Vis with a Waters 2487 dual lambda absorbance detector. The wavelength was set at 240 nm. The HPLC column was reverse-phase C18 Phenomenex Gemini 150×4.6 mm, 5 μm (Phenomenex, USA). The mobile phase composition was 30% acetonitrile and 70% 2.5 mM SDS in 0.01 M of citric acid. Melamine was eluted using an isocratic program; the mobile phase flow was 0.6 mL/min. An analytical standard of melamine was purchased from Sigma-Aldrich (USA). Five-point calibration was performed at the beginning of each sample batch. Quality control samples were blank and fortified blank feed samples (3 mg/kg b.m.). Recovery of the analytical method was 75%.

Results

Pathology

In all examined weaned piglets, necropsy revealed dehydrated and emaciated carcasses. The skin was pale, and the hair was rough. Lung oedema with foamy fluid in the trachea was observed in 4 out of 10 carcasses. The main macroscopic finding was observed on the kidneys. In all piglets, the kidneys were slightly enlarged, firm in consistency, the cortical surface was wrinkled, with a diffuse distribution of the yellow foci in the cortex and medulla (Fig. 1A). The renal capsule could be easily removed from the kidney surface. On the cut surface, yellow foci that looked like parallel lines were mostly observed in the cortico-medullary junction and renal crest (Fig. 1B). In addition, the renal pelvis was dilated, and the renal cortex and medulla were atrophied.

Microscopically, these yellow foci in the kidneys were accumulations of crystals predominantly located within the lumina of dilated distal straight and convoluted tubules and collecting ducts. (Fig. 2A). Some of these crystals appeared to be in the walls and lumina of blood vessels, in addition to the lumina of renal tubules. In four cases, renal tubules were disrupted, and the crystals were located in the adjacent interstitium. Marked distal tubular necrosis and intratubular crystals were present in all cases. In three cases, evidence of proximal tubular injury was also observed. The crystals measured up to approximately 20 to 100 μm in diameter. Larger crystals were more common in the medulla. Crystals were variable in shape and birefringent when viewed under polarized light. Two types of crystals were observed. The first type had dark striations radiating from the centre with an eosinophilic round centre and concentric lamina, while the second type of crystals was pale green to brown with irregular striated coarse granular aggregates and without the eosinophilic centre (Fig. 2B). Multi-focally, there were moderate aggregates of lymphocytes, plasma cells, macrophages and multinucleated giant cells within the interstitium (Fig. 2C). This finding was observed in all 10 cases. Moderate interstitial fibrosis was present in six out of ten cases, and it was clearly seen in sections stained with Masson's trichrome (Fig. 2D). Interstitial fibrosis and non-suppurative infiltrates indicated a chronic inflammatory response. Round centres in the first type of crystals, which were eosinophilic in HE staining, were PAS positive (Fig. 2E). In all ten cases, ORO72h staining revealed different degrees of positive staining in the first type of crystals (Fig. 2F). As in the PAS staining, ORO72h staining was exclusively found in the round centres of the first type of crystals.

Toxicological analysis

Melamine concentration in the pig milk replacer sample determined by HPLC/UV was 5 mg/kg of dry milk replacer. The milk replacer was added to the feed ration at 20%; therefore, the calculated final concentration of melamine in the daily feed ration was 1 mg of melamine per kg of feed mixture.

Discussion

Although, melamine is not approved for usage in animal feed, there is evidence of melamine toxicity, or melamine and cyanuric acid and melamine and its derivatives toxicity in pigs (3, 5, 6). In this study, poisoning of weaned piglets occurred accidentally, after using melamine-contaminated feed. The main clinical symptoms in post-weaning piglets were anorexia, wasting, polydipsia and lethargy. Similar symptoms were observed by Lee et al. (6). After the removal of the suspected feed, no further cases of illness were observed.

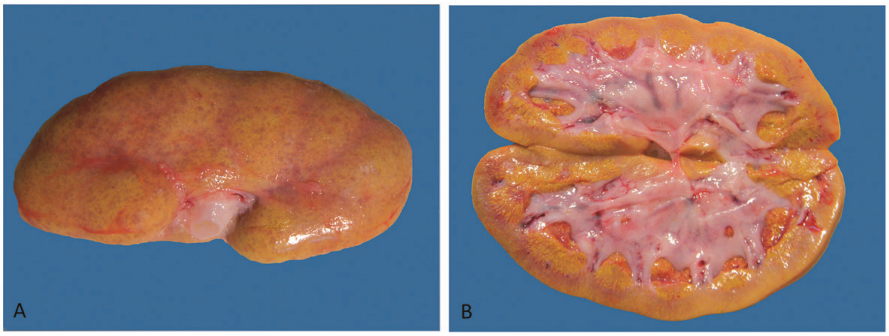

Figure 1: Gross lesions in kidneys after accidental melamine intoxication in weaned piglets. (A) Cortical surface of the kidney is yellowish and wrinkled. (B) Sagittal section of the kidney - diffuse distribution of the yellow foci in the cortex and medulla

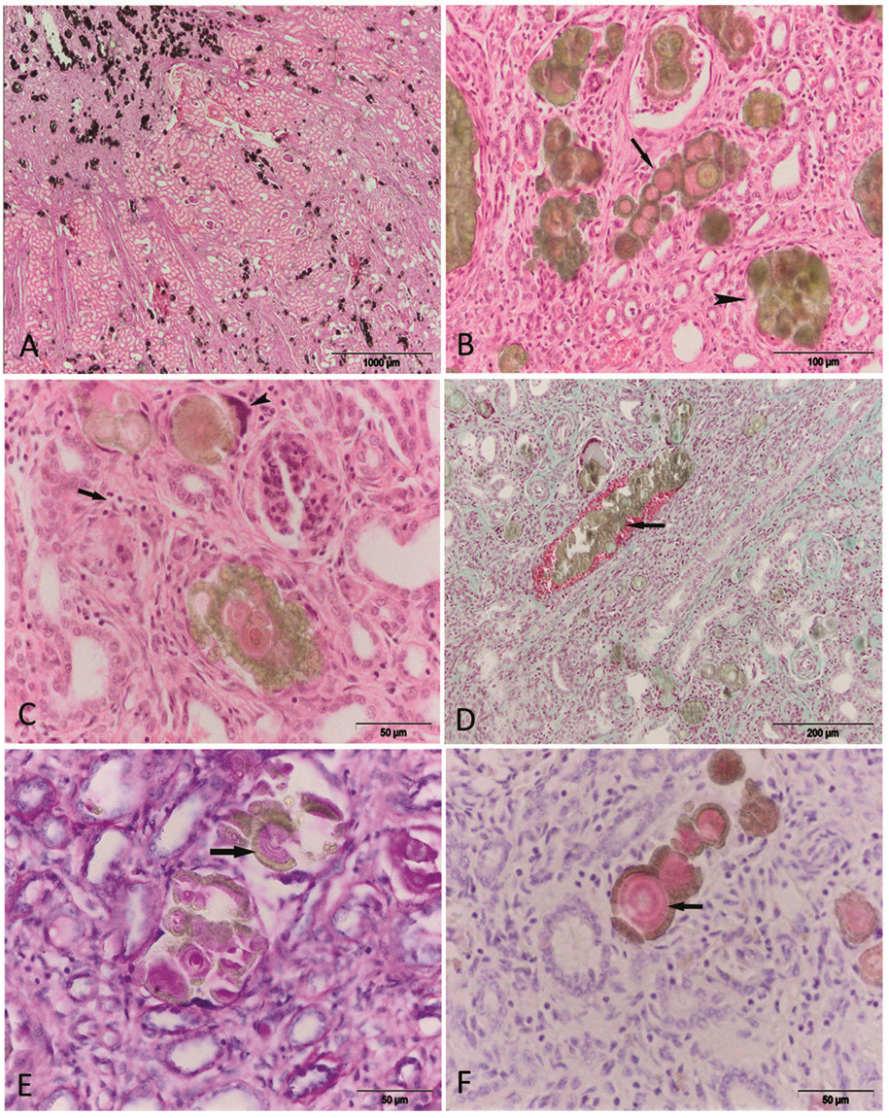

Figure 2: Microscopic lesions in accidental melamine intoxication in weaned piglets. (A) Accumulations of crystals predominantly within the lumina of dilated distal straight and convoluted tubules and collecting ducts. HE. (B) The first type crystals have several dark striations radiating from the centre with eosinophilic round centre (arrow), while the second type crystals are pale green to brown with irregular striated coarse granular aggregates and without eosinophilic centres (arrowhead). HE. (C) Moderate aggregates of lymphocytes, plasma cells, macrophages (arrow) and multinucleated giant cells around crystals within the interstitium (arrowhead). HE. (D) Moderate fibrosis – the renal interstitium is expanded by fibrous tissue (green). Crystal aggregates in blood vessel (arrow). Masson's trichrome. (E) PAS positive round centers in the first type crystals (arrow). PAS. (F) ORO72h positive round centres in the first type crystals (arrow). ORO72h.

The described macroscopic lesions in the kidneys are in accordance with findings described by Gonzales et al. (3) and Nilubol et al. (5). Wrinkled cortical surfaces, with yellow foci in the cortex and medulla can be characteristic lesions in melamine and/or cyanuric acid toxicosis in swine.

It is known that melamine is rapidly excreted in the urine and precipitated in the distal renal tubules (1), which is in accordance to our findings, showing accumulations of crystals predominantly within the lumina of dilated distal straight and convoluted tubules and collecting ducts. The first type crystals observed are similar to the crystals described by Thompson et al. (10) in three dogs with suspected pet food-induced nephrotoxicosis, and by Gonzales et al. (3) in swine, which were exposed to melamine and its derivatives. Similar crystals in melamine/cyanuric acid induced renal failure in dogs were described as "pinwheels" (11). Although ORO72h is primarily used for lipid staining, it is known from previous publications that prolonged staining with Oil Red O can be used for staining plastics (10), as we also found in our case in the first type crystals. Thompson et al. (10) found ORO72h positivity in melamine-containing crystals in dogs, and our results in swine are in accordance with their results. The first-type crystals were also positive to PAS staining. As the chemical structure of melamine includes three amino groups, it was thought likely that it would stain with PAS method.

Although melamine and cyanuric acid appeared to have low toxicity when administered separately, they induced extensive renal crystal formation when administered at the same time (12). In one investigation, crystals were also found in one of the two pigs fed 200 mg/kg body mass per day melamine only (13). Brand (4) found that the addition of cyanuric acid to a diet containing melamine caused less toxicity in poultry than a diet contaminated with only melamine. In our case, melamine was detected in the milk replacer, which was a component of feed for post-weaning piglets. It is possible that the melamine *per se* induced described pathomorphological changes, but the influence of other melamine derivatives cannot be excluded.

In this case, the diagnosis of nephrotoxicosis was based on the presence of crystals with characteristic light microscopic and histochemical properties, predominantly in the distal tubules and collecting ducts and subsequent demonstration of melamine in the milk replacer that was added to the complete feed. From the clinical signs, necropsy findings, and the histochemical and toxicological results, it could be concluded that melamine-contaminated feed was the most probable cause of nephrotoxicosis and death in the observed weaned piglets. Additionally, rather low concentrations (1 mg of melamine/kg of feed mixture) and a short period (3 weeks) of intake of melamine with feed may cause severe kidney damage, weight loss and death in weaned pigs. To our knowledge, this is the first report of poisoning due to melamine in weaned piglets in Serbia.

Acknowledgment

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NEFROTOKSIČNOST MELAMINA PRI ODSTAVLJENIH PUJSKIH V SRBIJI

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Povzetek: Živali sicer ni dovoljeno krmiti z melaminom, kljub temu pa obstajajo podatki o zastrupitvah s to snovjo. Če krma vsebuje melamin, lahko to povzroči nefrolitiazo, kronično vnetje ledvic, karcinom sečnika in celo pogin živali.

Septembra 2011 je v Srbiji na komercialni farmi za pitanje prašičev zbolelo 900 pujskov, starih 40 - 90 dni. Pri obolelih živalih so se pojavili anoreksija, hujšanje, polidipsija in letargija. Pri raztelesbi desetih pujskov so pri vseh ugotovili spremembe na ledvicah. Te so bile nekoliko povečane in čvrste konsistence, njihova površina je bila drobno nagubana, v skorji in sredici pa so bila difuzno razporejena rumena žarišča. Mikroskopsko so bila ta žarišča iz kristalov, nakopičenih v svetlini razširjenih premih, zavitih in zbirnih kanalčkov. Kristali so bili različne oblike in so lomili svetlobo pri opazovanju v polarizacijski svetlobi. Ugotovili smo dve vrsti kristalov: prvi tip je imel več temnih prog, ki so bile razporejene žarkasto okrog okroglega eozinofilnega središča, medtem ko je imel drugi tip bledo zelene do rjave nepravilno progaste agregate brez eozinofilnega središča. V vseh primerih smo v intersticiju ledvic ugotovili mnogožariščno razporejene skupine limfocitov, plazmatk, makrofagov in večjedrnih velikank.

Z analizo kliničnih znakov, ugotovitev pri raztelesbi, rezultatov histokemičnih in toksikoloških preiskav smo potrdili domnevo, da je bil glavni vzrok za nastanek sprememb v ledvicah in pogin pujskov krma, ki je bila kontaminirana z melaminom. Kolikor vemo, je to prvi opisani primer zastrupitve prašičev z melaminom v Srbiji.

Ključne besede: melamin; ledvica; odstavljeni pujski; nefrotoksičnost