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EXPOSURE TO MELAMINE FROM THE EARLY POSTNATAL PERIOD CAUSES NEPROTOXICITY: A HISTOPATHOLOGIC AND ULTRASTRUCTURAL STUDY

ERKEN POSTNATAL DÖNEMDEN İTİBAREN MELAMİN MARUZİYETİ NEFROTOKSİSİTEYE NEDEN OLUR: BİR HİSTOPATOLOJİK VE ULTRASÜTRÜKTÜREL ÇALIŞMA

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ABSTRACT

Objective: Melamine (mel), which is illegally added to formula to providing false-positive protein content, has caused acute renal failure in infants due to crystal formation. This study aimed to investigate the nephrotoxic effects of chronic low-dose mel exposure from the weaning period (supplementary food period).

Materials and Methods: Eighteen female rats in the weaning period (21-days-old) were divided into three groups. A 0.1 ml saline was given to the control group by oral gavage (p.o). Fifty mg/kg mel was given to the second group and 75 mg/kg mel to the third group dissolved in 0.1 ml saline for 21 days p.o.. At the end of the experiment, the animals were sacrificed, and histopathologic, morphometric, and ultrastructural analysis were performed on kidney tissues.

Results: There was an inflammatory cell infiltration in the tubulointerstitial area, and no crystal formation was observed in either of the mel groups. In the 75 mg mel group, glomerular and tubular epithelial damage and significant increases in Bowman's space were observed ($p<0.05$). In the ultrastructural analysis, the capillary lumen was closed due to endothelial enlargement, dilatation in the pedicles and hypertrophy in podocytes were found in the 75 mg group. Pedicles in the 50 mg group appeared to be enlarged more than the control group, but the capillary lumen was more open than the 75 mg group.

Conclusion: The results show that low dose mel exposure causes kidney damage with increased doses from the early postnatal period.

Keywords: Early postnatal period, electron microscopy, kidney, melamine, nephrotoxicity, rat

ÖZET

Amaç: İllegal olarak mamalara yalancı yüksek pozitif protein içeriği için eklenen melamin (mel), bebeklerde kristal oluşumuna bağlı akut böbrek yetmezliğine neden olmuştur. Bu çalışmada süt kesme döneminden (ek besin dönemi) itibaren kronik düşük doz mel maruziyetinin nefrotoksik etkilerinin araştırılması amaçlandı.

Gereç ve Yöntem: Süt kesim dönemindeki (21 günlük) 18 dişi sıçan üç gruba bölündü. Kontrol grubuna 0.1 ml serum fizyolojik oral gavajla (p.o) verildi. İkinci gruba 50 mg/kg mel, üçüncü gruba 75 mg/kg mel 0.1 ml serum fizyolojik ile çözülerek yirmi bir gün p.o. verildi. Deney sonunda hayvanlar sakrifiye edildi ve böbrek dokularında histopatolojik, morfometrik ve ultrasütrüktürel analiz yapıldı.

Bulgular: Her iki mel grubunda tübülointersitisyel alanda inflamatuar hücre infiltrasyonu vardı ve kristal oluşumu gözlenmedi. Yetmiş beş mg mel grubunda glomerüler ve tübüler epitel hasarı ve Bowman boşluğunda önemli artışlar gözlendi ($p<0.05$). Yetmiş beş mg grubunda ultrasütrüktürel analizlerinde endotel genişlemesi nedeniyle kapiller lümenin kapandığı, pedisellerde dilatasyon ve podositlerde hipertrofi saptandı. Elli mg grubundaki pediseller kontrol grubuna göre daha fazla genişlemiş gibi görünüyordu. Elli mg grubunda, kapiller lümen 75 mg grubuna göre daha açıktı.

Sonuç: Sonuç olarak, erken postanal dönemden itibaren düşük doz mel maruziyeti artan dozlarda böbrek hasarına neden olmaktadır.

Anahtar Kelimeler: Erken postnatal dönem, elektron mikroskopi, böbrek, melamin, nefrotoksisite, sıçan

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INTRODUCTION

Melamine (mel) is a chemical that has been illegally added to animal foods and formulas to show false high protein content. Indirect and unintentional mel exposure can happen due to agricultural insecticide such as cyromazine. After cyromazine contaminated vegetables are ingested in the body, it degrades to mel and cyanuric acid (cy) (1).

Melamin first attracted attention in 2007 by causing acute renal failure in animals due to contaminated pet foods (2). The mel-affected animals in 2004 and 2007 were analyzed and the presence of crystals and prominent lesions in the distal and collecting tubule were detected in their kidneys (3). After analysis of the pet food, it was noticed that it included cy and mel, and those chemical agents had caused crystal formation in the animal urine (4). In 2008, hundreds of thousands of infants suffered from renal failure due to crystal formation in the kidneys and several infants died after consuming mel-contaminated formula (5-7). According to the analysis of the contaminated formulas, they were contaminated only with mel, whereas pet food was contaminated with mel and cy (4). A previous study showed that the melamine+cyanuric acid (mel+cy) combination causes rapid crystal formation in animal urine, however, human studies have shown kidney stones formation caused by mel and uric acid (4). It reported that mel can't be metabolized and is excreted by the urinary system (8). Acute toxicity of mel is low and the median lethal dose (LD_{50}) of mel for oral exposure is 3828 mg/kg for female rats and 3161 mg/kg for males according to WHO reports in 2008 (9). Mel contamination attracted attention after the public health problem mentioned above, and pediatricians and nephrologists published a report on how to treat mel-induced renal failure (10). According to the literature, the mel+cy combination increases nephrotoxicity and crystal formation while mel alone has a less nephrotoxic effect. High doses or long term single mel exposure causes nephrotoxicity and males have been affected more negatively (11-17). The studies mostly focused on the mechanism of stone/crystal formation in kidney due to mel+cy exposure, the pharmacokinetics of mel, and effects of doses dependent mel exposure on the experimental animals (13, 16, 18, 19). However, Dalal et al. reported that formulas were contaminated by only mel, not mel+cy combination (4). Different from these animal experiments, a human study showed that even low-dose mel exposure in adults may increase the risk of urolithiasis (20, 21). There is still contradiction in the literature about the nephrotoxic effects of mel. According to a recent study, children and teenagers can also somehow be exposed to mel. Sathyanarayana et al. analyzed 109 children (4 months - 8 years) for urinary mel level and urinary markers of kidney injury. They found that mel levels in children were higher in the US than in other countries and increased in some urinary markers of kidney injury (22).

The early postnatal period (especially post-weaned period) is important because the supplementary food and formula consumption is higher and organs have not yet reached functional maturity. The studies on the nephrotoxic effects of low dose mel exposure in the early postnatal period, especially from the post-weaned period on female rats, are limited. This study aimed to investigate the nephrotoxic effects of low-dose mel exposure from the post-weaned period using histopathologic methods.

MATERIALS AND METHODS

This study was conducted in Giresun University Experimental Animals Research Laboratory with approval from the Giresun University Local Animal Ethics Committee (Date: 01.07.2019, No: 2019/11). The animal experiment was done in Giresun University Animal Research Centre. All 21-days-old female rats were kept in a light and dark environment for 12 hours at 22±2°C and 50±5% humidity. Standard pellet rat food and tap water were applied to 21-days-old weaned rats. The weaning period for rats is the postnatal 21st day (23). All animals were weighed daily and the dose was adjusted. Female rats (n=18, wistar albino, 30-40 g) were divided into 3 groups. Two different mel doses were applied in this experiment. According to an FDA report, 63 mg/kg bw/d (13 weeks, oral with feed, in rats) is the dose level without adverse effects (24). Dose levels were chosen higher and lower than 63 mg/kg, with reference to the FDA report and the 50 mg/kg and 75 mg/kg mel were applied to two different treatment groups. The first group received 0.1 mL saline by oral gavage (p.o.) for 21 days, the second group received 50 mg/kg/day mel (purity 99%, Sigma, Product Number: M2659) with 0.1 mL saline (p.o.) and the third group received 75 mg/kg/day mel with 0.1 mL saline (p.o.). On the 45th day, ketamine and xylazine (50 mg/kg and 5 mg/kg intraperitonally (i.p.) anesthesia was administered to prevent animals from suffering. The kidneys were removed, and the left kidney was put into neutral formalin (Interlab, 923.015.2500) for 48 hours. The right kidney was placed into 2.5% glutaraldehyde ((Merck, 1042390250, +4°C). After completing the experimental procedure, the rats were euthanized by high-dose anesthesia. After this automated tissue processing was made (Leica, ASP300S). Hematoxylin & Eosin (H&E) and Periodic Acid Schiff (PAS) staining were performed to show the changes in the glomerular and tubular structures and basement membrane, respectively.

H&E and PAS staining procedure

The left kidney tissues were passed through routine tissue processing and were embedded into paraffin (Thermo Scientific, 6774006). Two sets of 4 µm thick sections were taken from each paraffin block. After deparaffiniza-

tion, all sections from the first set were stained with H&E ((Sigma Aldrich 1043020025, Sigma Aldrich, E4009) and were passed through increased alcohol series (70, 80, 96, 99 %) for dehydration and xylene. Then all sections were covered.

The second set sections were subjected to the deparaffinization process (xylene and descending ethyl alcohol series). Sections were kept in 0.5% periodic acid solution (10 min.) (Merck, 1005240025) and then in Schiff reagent solution (20 min.). Then, these sections were washed with sodium metabisulphite for 2x5 minutes and stained with Harris hematoxylin. After dehydration, the sections were closed with entellan®. Figures captured from the slices in Zeiss Imager A-2 Axio (Germany) computer-aided light microscope were evaluated in Zeiss ZEN imagine software. In the H&E stained sections, Bowman's space and Bowman capsule diameter, and glomerular area were measured in the random 10 glomerul from each animal at x20.

Tissue preparing for transmission electron microscopy (TEM)

The kidney tissues were fixed in 2.5% glutaraldehyde (Merck, 1042390250), (+4°C) in 0.1 M phosphate buffer for 4 h and postfixed with 1% OsO4 (Merck, 1245050001) for 1 h (+4°C). Block contrast was performed for 1 hour (+4°C) in 1% Uranyl acetate (EMS, 22400). It was then dehydrated with an increasing series of ethanol (70, 80, 90, 96, 100%). Ethyl alcohol was removed by applying propylene oxide twice and then tissues were embedded in epoxy resin (Sigma Aldrich, 45359). 60 nm thickness sections were taken with Leica EM UC7 ultramicrotome. Five percent Uranyl acetate and Reynold's solutions were applied for contrast. The analysis was performed using the JEOL 1011 Transmission electron microscope (80 mV).

Statistical analysis

Statistical analysis was performed with SPSS Statistics 22. The One Way ANOVA test was used to analyse and interpret the data and the Tukey test was used to decide differences between the groups. Values at $p<0.05$ were accepted to be statistically significant.

RESULTS

Histopathologic evaluation

According to the analysis of H&E staining, glomeruli, proximal and distal tubule structures were observed as being normal with no presence of tubular crystals or inflammatory cell infiltration in the control group (Figure 1A, 2A). The basement membrane in the glomerulus,

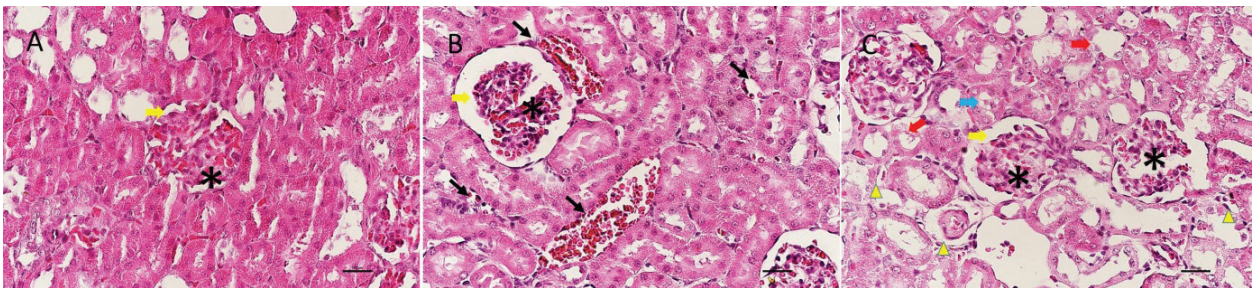

Figure 1: Glomerular and tubular structure in the kidney sections of all groups, H&E, x 40, scale bar: 100 µm, A: Control group (star: glomerulus, yellow arrow: Bowman's space), B: 5 0 mg mel group, (star: glomerulus, yellow arrow: Bowman's space, black arrow: dilatation of vessels and congestion), C: 75 mg mel group, (star: glomerulus, yellow arrow: Bowman's space, blue arrow: vacuole, red arrow: degeneration epithelium, arrowhead: widening in interstitium and edema)

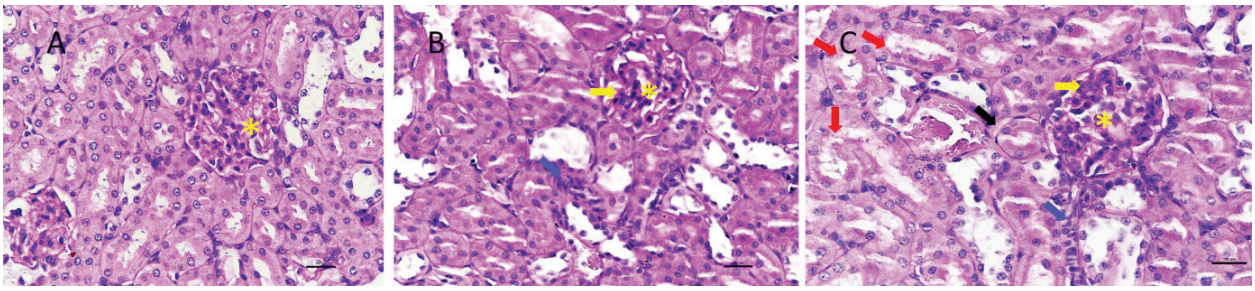

Figure 2: Kidney sections of experimental groups, PAS, x 40, scale bar: 100 µm, A: Control group (star: glomerulus), B:50mg mel group, (star: glomerulus, blue arrow: inflammatory cell infiltration in the interstitium, yellow arrow: increased mesangial matrix and glomerular basement membrane thickening), C: 75 mg mel group (star: glomerulus, yellow arrow: increased mesangial matrix and glomerular basement membrane thickening, red arrow: impaired brush border, black arrow: lack of basement membrane, blue arrow: inflammatory cell infiltration in the interstitium)

basement membrane proximal tubule, and distal tubule, and epithelium were not thickened in PAS staining (Figure 2A). In the 50 mg mel group, glomeruli, proximal and distal tubule structures were observed as being normal and there was no presence of tubular crystal. However, there was a slight increase in the Bowman's space and dilatation of vessels, and congestion of the intertubular area (Figure 1B). Inflammatory cell infiltration draws attention in the tubulointerstitial area (Figure 2B). According to the PAS staining, the basement membrane of proximal and distal tubules were observed as being normal, however, an increase of the mesangial matrix and thickness of the glomerular basement membrane was observed in the 50 mg mel group (Figure 2B). In the 75 mg mel group, an increase of the Bowman's space, disruption in the glomerular integrity and tubular epithelium, dilatation of proximal and distal tubules, vacuolization of tubule epithelium, diffuse inflammatory cell infiltration, widening in the interstitium and edema were observed in the tubulointerstitial area (Figure 1C, 2C). According to the PAS staining, partial disruption in the proximal basement membrane, an increase of mesangial matrix, and thickness of glomerular basement membrane were observed (Figure 2C).

Evaluation of morphometric measurements

The mean size of the glomerular area was 17916.490±728.270 μm^2 in the control group. In the 50 mg mel group, the mean size of the glomerular area was 16635.620±677.630 μm^2, it was 16264.150±615.292 μm^2 in the 75 mg mel group. According to the statistical results, although there was a decrease in the glomerular area related to an increased dose in both mel treatment groups, no statistically significant difference was found between both mel groups and the control group (p>0.05) (Figure 3)

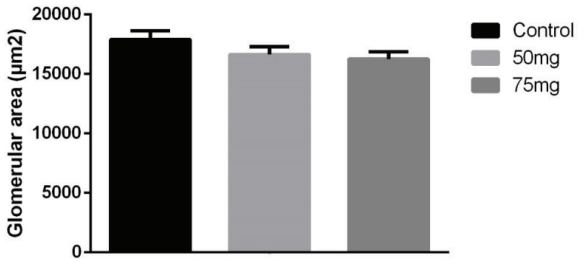

Figure 3: Glomerular area measurements of groups (μm^2)

The mean length of the Bowman's space was 12.087±0.513 μm in the control group. In the 50 mg mel group, the mean length of the Bowman's space was 14.143±0.565 μm, it was 19.057±0.928 μm in the 75 mg mel group. According to the results for Bowman's space length, there was an increase in Bowman's space in the 50 mg mel group compared to the control group, but it was not statistically significant (p>0.05). The difference between the 75 mg and 50 mg mel groups and between the 75 mg mel group and control groups in terms of length measurement of Bowman's space was statistically significant (p<0,05) (Figure 4).

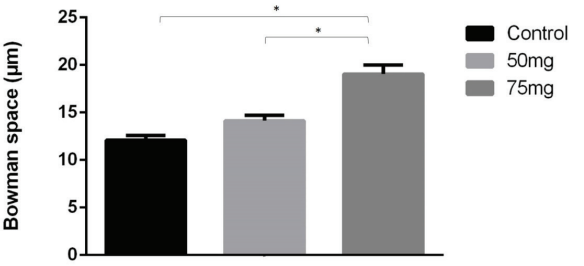

Figure 4: Bowman's space measurements of groups (μm)

The mean Bowman capsule diameter was 79.54±1.47 μm in the control group, it was 79.59±1.89 μm in the 50 mg mel group and 80.61±1.37 μm in the 75 mg mel group. According to the statistical results, there was no statistically significant difference found between both mel groups and the control group (p>0.05) (Figure 5)

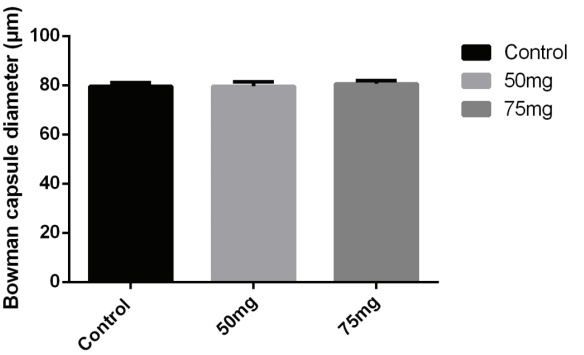

Figure 5: The measurement of Bowman's capsule diameter of groups (μm)

Ultrastructural analysis

Normal pedicel and capillary appearance were observed in the control group (Figure 6A). In the 50 mg mel group, the pedicels seemed to have expanded compared to the control group, but the pedicle enlargement was less than the 75 mg group. The capillary lumen was more open than the 75 mg group (Figure 6B). In the 75 mg group, extraordinary endoplasmic reticulum was seen in the endothelial cells. The capillary lumen was closed due to endothelial enlargement (Endotheliosis). Pedicle enlargement (effacement) was observed. Podocytes were hypertrophic. Mesangial matrix increase was observed (Figure 6C).

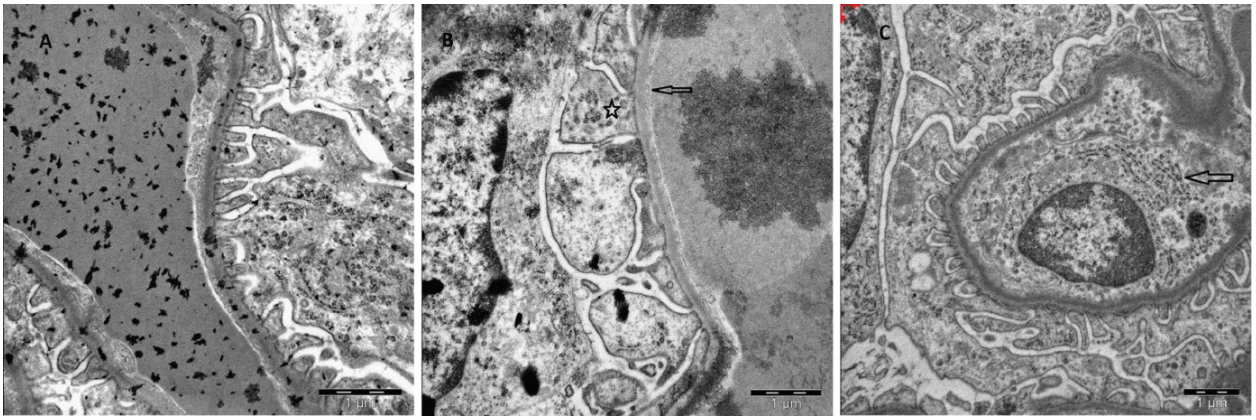

Figure 6: Kidney sections of the experimental groups, TEM, A: The sections of the control (SF) group has normal pedicel and capillary appearance, (X 7 5000), B: In the glomerulus of the 50 mg group, pedicels appear enlarged (star). (X 7 500). Endothelial fenestration (arrows), C: Endoplasmic reticulum (arrow) is seen in an endothelial cell filled a capillary lumen in the 75 mg group. (X 7 500).

DISCUSSION

Mel-contaminated food consumption has previously caused kidney failure in babies. The experimental studies focused on nephrotoxic effects of mel at high doses and the crystal-forming mechanism. The current study focused on the nephrotoxic effects of low dose mel exposure during the post-weaning period and the results showed three weeks of 50 mg and 75 mg mel exposure from the early postnatal period caused inflammation, whereas 75 mg mel exposure gave rise to kidney damage and a significant increase in the Bowman's space width. Even if the reduction in the glomerular area wasn't statistically significant, 75 mg mel exposure caused a decrease in the glomerular area. This result shows that 75 mg mel may cause degeneration in the podocytes or mesangial cells. Morphometric changes of the glomerulus and Bowman's space width can indicate various kidney diseases (25). According to a Food and Drug Administration (FDA) report in 2008, 63 mg/kg bw/d equated to no observed adverse effect level in rats (13 weeks p.o.) (24). Similarly, Park et. al. applied 50 mg mel for 3 days to 7-weeks-old male Spraque Dawley rats and it didn't cause crystal formation or toxic lesions in renal tubules (11). In parallel with the literature, structural damage may start between 50 mg and 75 mg. However, inflammatory cell infiltration in the intertubular area were observed at 50 mg mel exposure, which was less than 63 mg and LD_{50}.

Another important point of the present study is the exposure period. Because the mel-exposed age group during the food scandal was mostly in the baby period, that is why post weaned rats were used in the present study. The efficiency and ability of kidney functions and structure are different in the early postnatal and adult period. It does not have sufficient function until postnatal 6 weeks for rats and until postnatal 18 months for humans (26). For this reason, any toxicological agent may negatively affect immature kidney function and structure. Similarly, Yasui et al. tried to show mel effects on the different exposure age groups. In the study 6-, 10-, and 26-weeks-old F334/N rats were exposed to 12 mg mel+cy for 28 days. According to the results, the kidneys of the 6-weeks-old group was affected more negatively (12). It seems that mel exposure during the developing period mostly causes nephrotoxicity. In the literature, animal experimental studies have mostly been conducted with adult age group rats.

In the literature, there is controversy about the nephrotoxic effects of single mel exposure. Even if previous studies showed that increased doses of mel exposure cause nephrotoxicity, some of them have shown that mel exposure alone may not be nephrotoxic. Xie et al. administrated 100, 300, and 600 mg doses of mel, cy (100 mg), and mel+cy 50 mg +50 mg by oral gavage for 15 days, and they showed that these mixtures and the highest mel dose has renal toxicity (15). Some previous studies showed that even only high dose mel usage couldn't cause nephrotoxicity. Jacob et al. applied 7, 23, 69, 229, or 694 ppm mel+cy combination, 1388 ppm mel, and 1388 ppm cy to male and female F334/N rats for seven days. Only mel or cy treatment didn't cause any toxicity, and 229 or 694 ppm mel+cy combination caused a significant increase in blood urea nitrogen and serum creatinine levels and crystal formation (13). Peerakietkhajorn et al. showed acute toxicity in Wistar rats treated with 400 mg mel, and 400 mg combination of mel+cy for three days. The results indicated that a mixture of mel+cy can cause glomerular atrophy and renal tubular dilation, affecting the function of the kidney, such as sodium and potassium secretions, while mel alone did not affect kidney structure

and function in the acute toxicity test (14). In the present study, the mel exposure dose was quite low compared to those studies, however, exposure time and period in the present study was the notable part of a comparison with the mentioned studies. The reason for the nephrotoxic effects of the low dose and long period mel exposure may be increased intracellular ROS (reactive oxygen species) and apoptosis. Guo et al. showed by an in vitro study that rat NRK-52e kidney epithelial cell line exposure 24 μM mel for 24 h causes increase intracellular ROS (reactive oxygen species) and apoptosis by the activation of p38 MAPK pathway (27). Kuo et al. showed an in vitro study that mel exposure on human embryonic kidney cell line HEK293 increased ROS (28). Lee et al. showed that a mixture of cy and mel increases nephrotoxicity by reducing antioxidant enzyme activities, increases apoptosis tubular cells (increased Bax level, decreased Bcl-2 level, caspase-3 activation) (29).

Another common finding of mel exposure is inflammation. Kuo et al. administered mel macrophage cell line and human embryonic kidney cell line at varying doses (1 pM, 1 nM, and 1 μM for 1 or 24 h) and showed that inflammation and oxidative stress increased by NF-κB/COX-2 and NOX/ROS pathway (28). Zhou et al. showed increased immunoglobulin M levels and decreased levels of $CD3^+$, $CD4^+$ in a study with 170 children with stones who had consumed mel-contaminated powdered formula. However, there was no difference in blood count between the children with kidney stones and without kidney stones (5). Although the presence of inflammation is parallel to previous studies, it has been demonstrated in the current study that low-dose exposure may cause inflammation. In our previous study (30), blood analysis showed that 75 mg mel exposure causes lymphocytosis and increases the number of white blood cells (30).

Beside the animal experimental studies, a clinical study reported that mel exposure may still be a public health problem for children. Sathyanarayana et al showed that children can be exposed to mel from different sources. In their study, 109 children (4 months - 8 years) were analyzed for urinary mel level and urinary markers of kidney injury. They found that mel levels in children were higher in the US than in other countries and increased in some urinary markers of kidney injury (22).

Although high-dose mel has been shown to cause nephrotoxicity in the literature, it was shown in the current study that low-dose mel exposure from the post-weaned period can also cause nephrotoxicity. Although Langman et al. published a report on how to treat mel-induced renal failure for pediatricians and nephrologists (10), attention should be drawn to low-dose and long-term mel exposure.

There is a limitation of this study - according to the present study above 75 mg seems to cause kidney damage, in parallel with the FDA report. However, it is not known at what dose level between 50 and 75 mg the nephrotoxic effect begins. Dose studies are needed to determine at what dose and at what exposure time melamine nephrotoxicity begins for the early postnatal period.

CONCLUSION

The results show that low-dose mel exposure from the early postnatal period causes inflammation and structural damage in rat kidneys. Based on this result, illegally mel-contaminated formula is a potential pediatric health problem due to nephrotoxic effects. Not only human nourishment is affected - animals may face the same potential risk because of mel-contaminated pet foods.

Ethics Committee Approval: This study was approved by Giresun University Faculty of Health Sciences Ethics Committee (Date: 01.07.2019, No: 2019/11).

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