

Evaluation of melamine and cyanuric acid cytotoxicity: an in vitro study on L929 fibroblasts and CHO cell line

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Abstract: Melamine and its metabolites pose health concern as they are used in various industrial products including feed and drugs. There are a limited number of studies on melamine and cyanuric acid cytotoxicity and cellular damage without a certain conclusion. The present study aimed to evaluate melamine, cyanuric acid and its combined cytotoxic effects using 3-(4,5-dimethylthiazol-2-yl) methyl thiazole tetrazolium (MTT) bromide test. The study also evaluated apoptotic and necrotic effect using a double staining method of Hoechst 33342 and propidium iodide. Melamine, cyanuric acid and their combination (1:1) were applied to L929 fibroblasts and Chinese hamster ovary (CHO) cells at various concentrations (1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL and 62.5 µg/mL). At the highest concentration (1000 µg/mL), the cell viability dropped down approximately to 50% both in CHO cells and L929 cells. Melamine, cyanuric acid and their mixture caused cytotoxicity in CHO cells and L929 fibroblasts in dose-dependent manner. Cell death occurred through both apoptosis and mainly necrosis. Both cell types were more sensitive to the mixture of melamine and cyanuric acid and, furthermore, CHO cells were more sensitive than L929 fibroblasts. As a result, melamine, cyanuric acid and their combination caused cytotoxicity in CHO cells and L929 fibroblasts. Further studies should be conducted in different cell lines. These studies should also aim to reveal the mechanism of cytotoxicity and related pathways.

Keywords: Apoptosis, cyanuric acid, cytotoxicity, melamine, necrosis.

Melamin ve siyanürik asit sitotoksitesinin değerlendirilmesi: L929 fibroblast ve CHO hücre hattı üzerine *in vitro* çalışma

Özet: Melamin ve metabolitleri yiyecek ve ilaç gibi farklı endüstriyel ürünlerde kullanılması sebebiyle sağlık endişesine neden olmaktadır. Melamin ve siyanürik asit sitotoksitesi ve bununla ilgili hücre hasarı üzerine çok az sayıda çalışma olup, bu çalışmaların hiçbirisinde de nihai bir sonuca varılamamıştır. Bu çalışmada, 3-(4,5-dimethylthiazol-2-yl) metil tiazol tetrazolium (MTT) bromür testi kullanılarak melamin, siyanürik asit ve bu ikisinin kombinasyonunun sitotoksik etkilerinin değerlendirilmesi amaçlandı. Çalışmada aynı zamanda, Hoechst 33342 ve propidyum iyodürün çift boyama yöntemini kullanarak apoptotik ve nekrotik etkileri de değerlendirdi. Melamin, siyanürik asit ve bunların kombinasyonları (1:1) L929 fibroblastlarına ve CHO hücrelerine çeşitli konsantrasyonlarda (1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL and 62,5 µg/mL) uygulandı. En yüksek konsantrasyonda (1000 µg/mL), hücre canlılığı hem CHO hücrelerinde hem de L929 hücrelerinde yaklaşık %50'ye düştü. Melamin, siyanürik asit ve bunların karışımı, CHO hücrelerinde ve L929 fibroblastlarında doza bağlı sitotoksiteye neden oldu. Her iki hücre de melamin ve siyanürik asit karışımına daha duyarlı ve ayrıca CHO hücreleri, L929 fibroblastlarına göre daha hassas olarak bulundu. Hücre ölümü, apoptoz ve esas olarak da nekroz yoluyla oluştu. Sonuç olarak, melamin, siyanürik asit ve bunların kombinasyonu CHO hücreleri ve L929 fibroblastlarında sitotoksiteye neden olmaktadır. Başka hücre hatlarıyla da ilave çalışmaları yapılmalıdır. Bu bu çalışmalar sitotoksitesinin mekanizması ve ilgili hücre yollarının aydınlanmasını amaçlamalıdır.

Anahtar sözcükler: Apoptoz, siyanürik asit, sitotoksite, melamin, nekroz.

Introduction

Melamine (2,4,6-triamino-1,3,5-triazine, C₃H₆N₆) is a synthetic chemical used in various fields of industry

for production materials including plastics, adhesives, fertilizers, housewares, and others (29, 34, 37). As a sanitizing agent, melamine is also used in food processing

equipment and instruments (11, 33, 37). Melamine was also investigated for its anticancer potential activity, but the related results resulted in a lack of efficacy (18, 37). Besides these useful potentials, melamine toxicity is one of the major concerns, especially in the food industry. Acute melamine toxicity is generally considered to be low in animals and frequently targets kidney (5, 20). However, a number of recent studies concluded that pathologies resulting from melamine-related toxicity are not limited to renal tissues (15, 36). It was recorded that chronic exposure to melamine may cause reproductive damage to the eye, skin and respiratory system (28, 42). As a consequence of melamine ingestions especially at higher doses, melamine causes testicular toxicity in mice with a significant rate of sperm abnormality and DNA damage (36, 38, 43).

Cyanuric acid (1,3,5-triazine-2,4,6-triol; CAS No. 108-80-5) is a structurally related constituent to melamine and frequently used in a ruminant diet as a feed additive and chlorine-stabilizing agent (11, 19, 21). As a structural analogue of melamine, cyanuric acid alone exhibits rather low acute toxicity (22, 24). Cyanuric acid has a strong mutual affinity to melamine and it has been reported that the melamine and cyanuric acid mixture or its combined effect was held responsible for the renal injury (22, 26). In the presence of cyanuric acid, melamine forms hydrogen bonds with cyanuric acid that results in the formation of an insoluble crystal named melamine cyanurate (10). A number of recent research concluded that melamine with or without cyanuric acid seems to be toxic to tissues and organ systems including testis, spermatozoid (6, 36, 43), liver, stomach, small intestine (7, 36) and hippocampus (1, 2). In a previous study by Yin et al. (37) concluded that ingestion of melamine alone or its combination with cyanuric acid had certain toxic effects on mouse spleen (39). A recent rat study concluded that melamine passes through the placenta in a dose-dependent manner (9, 17). Thus, melamine toxicity pose treats to unborn babies as they are more sensitive to chemical insults (3). Both melamine and cyanuric acid even at low-doses can interfere with the blood-testis barrier in mouse (6). An et al. (1) reported that the melamine toxicity in the nervous system is selective for the hippocampus in which melamine can induce the free radical formation and lipid peroxidation. Another study by An and Zhang (2) investigated prenatal exposure to melamine that resulted in impaired synaptic activity in the hippocampus (38).

Although limited in number, *in vitro* studies have also revealed melamine cytotoxicity using various cell lines, e.g. differentiated PC12 cells and mouse spleen lymphocytes (14, 37). Partanen et al. (23) observed decreased cell viability of trophoblast derived BeWo cells in response to melamine exposure with or without the presence of cyanuric acid. Cell lines vary in response or

sensitivity to melamine and cyanuric acid exposure. For instance, Choi et al. (8) reported that CHN human renal adenocarcinoma cell line was more sensitive to melamine, cyanuric acid or their combination compared to MDCK canine kidney epithelial cell line. In a study by Radko et al. (25), hepatoma (line FaO) seems to be more sensitive than rat skeletal muscle (line L6) to melamine. Thus, there is a growing interest in characterizing cytotoxicity of melamine, cyanuric acid or their mixture on different cell lines.

The mechanism of melamine and cyanuric acid cytotoxicity or activity has also been one of the major scientific interests. Melamine alone or its combination with cyanuric acid resulted in liver damage in mice in a dose-dependent manner. The damaged liver areas were characterized by apoptosis in hepatocytes with up regulation of apoptosis-related genes, Bax, cyt-c and caspase-3 (7, 35, 36, 40). Yiu et al. (41) reported that melamine induced apoptosis in the porcine renal proximal tubule cell line, LLC-PK1, through Ca²⁺-sensing receptor activation. Another study by Gohel et al. (12) found that melamine cyanurate damaged the integrity of tight junction when crystals were physically formed on the apical cell surface. It also causes oxidative DNA damage, in addition to up-regulation of inflammatory cytokines including IL-5, IL-6, IL-8, and MCP-1. Thus, there is a growing scientific interest to reveal the degree of cell viability in different cells and the mechanism of cell death as a consequence of exposure to melamine with the presence or absence of cyanuric acid.

In the present study, we aimed to investigate cytotoxic, apoptotic and necrotic effects of melamine and cyanuric acid and their combination on well characterized two cell lines, mouse L929 fibroblasts and CHO cells, an epithelial cell line derived from the Chinese hamster ovary.

Material and Methods

Determination of cytotoxicity using MTT test: MTT is a very sensitive test to determine cell proliferation and cell viability using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) salt. L929 fibroblasts and Chinese hamster ovary (CHO) cells were inoculated into 96 well plates at a concentration of 10×10^3 per well. Cells were incubated for 24 hours in an incubator conditioned with 5% CO₂ at 37 °C. First, 10 mg of melamine and cyanuric acid were poured into separate tubes and 1 ml of 10% DMSO was added and sonicated afterwards. Then, 9 ml of DMEM medium was also added to each tube and vortexed. Then, melamine (99%, Sigma–Aldrich, USA), cyanuric acid (98%, Sigma–Aldrich, USA) and their combination (1:1) were applied to L929 fibroblasts and CHO cells at various concentrations (1000 µg/mL, 5090 µg/mL, 250 µg/mL, 125 µg/mL and

62.5 µg/mL) and incubated for 24 hours. Samples were studied in triplicate. For control, only cell medium was used. At the end of 24 hours, cell medium was removed from the wells and 50 µL MTT solution was added to each well. Cells were incubated with MTT solution for 2 hours. Upon draining the MTT solution, 100 µl of isopropanol was added into each cells. Then, it was shaken gently and read in an ELISA plate reader at 570 nm wavelength. The final data of cell viability (%) were obtained by comparing the results obtained from the ELISA reader to that of control.

Determination of apoptosis and necrosis using double staining method: The double staining cocktail was prepared as follows: Ribonuclease-A stock solution was prepared as it contained 10 mg of RNase in 1ml PBS. The Hoechst 33342 and PI stock solutions were prepared as it contained 200 µg of Hoechst 33342 and 100 µg of PI in 1 ml of PBS. The working solution was prepared as follows: 100µL of RNase and 500 µL Hoechst, and 100µL of PI stock solutions were added into 10 mL PBS.

L929 fibroblasts and CHO cells were inoculated into 48 well plates at a concentration of 10×10^3 per well. Cells were incubated for 24 hours in an incubator conditioned with 5% CO₂ at 37 °C. Then, melamine, cyanuric acid and their combination (1:1) were applied to L929 fibroblasts and CHO cells at various concentrations (1000µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL and 62.5 µg/mL) and incubated for 24 hours. Samples were studied in triplicate. For control, only cell medium was used. At the end of 24 hours, cell medium was removed from the wells and 79 µL of the working double staining solution was added to each well. The 48 well plates were incubated in an incubator for 15 min. For this 15 min incubation period, a completely dark environment is required. At the end of incubation, cells were evaluated in a fluorescence

microscope. Apoptotic and necrotic cells were visualized using the DAPI and FITC (480-520nm wavelength) fluorescence filters, respectively.

Statistical analysis: For statistical analyses, the presence of difference was examined using the one way analysis of variance (ANOVA). In case of presence of a significant difference, the Tukey test was conducted for multiple comparisons. For comparison between CHO cell and L929 fibroblast for the same dose of the same chemicals, the student's *t*-test was used. Data were expressed as mean ± standard deviation. A *P* value of <0.05 was considered statistically significant.

Results

MTT test: Based on ELISA plate reader, the cell viability (%) results on L929 fibroblasts and CHO cells were given in Table 1. The cell viability in L929 fibroblasts and CHO cells after application of melamine, cyanuric acid, and their mixture decreased remarkably in a dose dependent manner. The cell viability on L929 fibroblasts was the lowest at the highest dose used (1000 µg/mL) without any significance to any group (*P* > 0.05). The cell viability rates on L929 fibroblasts after application of melamine, cyanuric acid and their mixture at a dose of 1000 µg/mL were 49.40±1.14%, 51.20±1.30, and 47.80±1.30% respectively. Beginning at the dose of 1000 µg/mL, the cell viability was significantly lower in L929 fibroblasts treated with mixture of melamine and cyanuric acid compared to those treated either with melamine or cyanuric acid alone (*P* < 0.05). For instance, the cell viability rates on L929 fibroblasts following application of melamine, cyanuric acid and their mixture at a dose of 62.5µg/mL, were 100.60±1.14%, 98.20±1.30 %, and 78.20±0.83 respectively (*P* < 0.05).

Table 1. Cell viability (%) of CHO cells and L929 fibroblasts upon exposure to melamine, cyanuric acid and their mix (1:1)

Concentration (µg/mL)		Melamine	Cyanuric acid	Melamine-Cyanuric acid
1000µg/mL	CHO cells	48.40±0.54 ^{a*}	48.20±1.30 ^a	41.20±0.83 ^b
	L929 Fibroblasts	49.40±1.14 ^{ab}	51.20±1.30 ^a	47.80±1.30 ^b
500 µg/mL	CHO cells	64.60±1.14 ^{b*}	84.60±1.14 ^{a*}	54.60±1.14 ^{c*}
	L929 Fibroblasts	79.20±1.64 ^a	80.20±1.78 ^a	51.40±1.34 ^b
250 µg/mL	CHO cells	77.60±1.14 ^{b*}	86.40±1.51 ^{a*}	56.80±0.83 ^{c*}
	L929 Fibroblasts	96.20±3.0 ^a	95.60±5.1 ^a	52.60±2.7 ^b
125 µg/mL	CHO cells	82.40±1.14 ^{a*}	91.20±1.30 ^{a*}	68.60±1.14 ^{b*}
	L929 Fibroblasts	97.80±0.83 ^b	95.80±1.09 ^a	62.80±1.30 ^c
62.5 µg/mL	CHO cells	85.80±0.83 ^{a*}	92.80±1.30 ^{a*}	69.80±0.83 ^{b*}
	L929 Fibroblasts	100.60±1.14 ^b	98.20±1.30 ^a	78.20±0.83 ^c
Control			100	

Note: Data with different letters in superscript in the same row is statistically significant (*P* < 0.05). Presence of an asterisk (*) indicates a significant difference in the same column for the same dose (*P* < 0.05)

The cell viability on CHO cell was the lowest at the highest dose used (1000 µg/mL) and it was lower in cells treated with the mixture of melamine and cyanuric acid ($P>0.05$). The cell viability rates on CHO cell after application of melamine, cyanuric acid and their mixture at a dose of 1000 µg/mL were 48.40 ± 0.54 , 48.20 ± 1.30 , and $41.20\pm0.83\%$ respectively. In MTT cells, the order of cytotoxicity induction in CHO cells from highest to lowest was as follows: the mixture > melamine > cyanuric acid. Such a distinction was the most prominent especially at a dose of 500 µg/mL and 250 µg/mL ($P<0.05$) although the cell viability was always lower in CHO cells exposed to the mixture ($P<0.05$).

Table 1 also compares the difference between CHO cells and L929 fibroblasts for the cell viability following exposure to various concentrations of melamine, cyanuric acid and their mixture. CHO cells seem to be more sensitive to all. The difference was significant at all concentrations of melamine, cyanuric acid and the mixture ($P<0.05$), except at the highest concentrations of cyanuric acid and the mixture.

Apoptotic and necrotic index: Apoptotic cells were determined under a fluorescence microscope using the

DAPI filter. The apoptotic cell nuclei were identified as they illuminate bright shining blue color (Figures 1 and 2). Apoptotic rates in L929 fibroblasts and CHO cells exposed to melamine, cyanuric acid and their mixture were given in Tables 2. The apoptotic index in both CHO cells and L929 fibroblasts is limited and there was no significant difference in comparison of cytotoxicity either of substances, except at 500 µg/mL and 125 µg/mL concentrations of melamine applied on to CHO cells. In comparison between CHO cells and L929 fibroblasts, the significant difference occurred at 125 µg/mL of cyanuric acid and the mixture. The necrotic index in both kinds of cells increased in a dose dependent manner (Table 3). The mixture of melamine and cyanuric acid mixture caused the highest necrotic effects, specifically at 500 µg/mL, 250 µg/mL and 125 µg/mL ($P<0.05$). There was no statistically significant difference between CHO cells and L929 fibroblasts by means of the necrotic rate following exposure to any substances used ($P>0.05$). The double staining of L929 cells and CHO cells were depicted in photomicrographs in Figures 1 and 2, respectively.

Table 2. The apoptotic index (%) resulting from exposure of CHO cells and L929 fibroblasts upon exposure to melamine, cyanuric acid and their mix (1:1)

Concentration		Melamine	Cyanuric acid	Melamine-Cyanuric acid
1000 µg/mL	CHO cells	5±1.58	5.8±1.92	8±2.54
	L929 Fibroblasts	6.6±1.51	5.2±1.09	7±2.54
500 µg/mL	CHO cells	4±0.70 ^a	4.6±1.14 ^a	7±2.23 ^b
	L929 Fibroblasts	5.4±1.14	5.8±1.48	6.4±2.07
250 µg/mL	CHO cells	3.2±0.83	4±0.70	4.6±1.14
	L929 Fibroblasts	4.4±1.34	3.4±1.14	4.4±0.89
125 µg/mL	CHO cells	2.4±0.54 ^a	4.6±0.89 ^b	3.8±0.83 ^b
	L929 Fibroblasts	2.6±0.89	2±1.41 [*]	2.2±0.83 [*]
62.5 µg/mL	CHO cells	1.6±0.89	2±0.70	3±1.00
	L929 Fibroblasts	1.6±0.54	1.6±0.89	2.6±0.54

Note: Data with different letters in superscript in the same row is statistically significant ($P<0.05$). Presence of an asterisk (*) indicates a significant difference in the same column for the same dose ($P<0.05$)

Table 3. Necrotic index (%) in CHO cells and L929 fibroblasts upon exposure to melamine, cyanuric acid and their mix (1:1).

Concentration		Cyanuric acid	Melamine	Melamine-Cyanuric acid
1000 µg/mL	CHO cells	33±4.94	36.4±3.64	40.2±4.81
	L929 Fibroblasts	38±2.86	34±4.12	37±4.35
500 µg/mL	CHO cells	18.8±5.76 ^a	24.8±7.66 ^{ab}	33±4.30 ^b
	L929 Fibroblasts	16.4±3.64 ^a	19.80±5.71 ^a	28.8±4.65 ^b
250 µg/mL	CHO cells	14.8±3.19 ^a	16.8±5.16 ^a	28.4±4.77 ^b
	L929 Fibroblasts	12±2.73 ^a	13.6±7.19 ^a	25±5.09 ^b
125 µg/mL	CHO cells	7.2±3.96 ^a	7.6±2.40 ^a	14±3.39 ^b
	L929 Fibroblasts	7±4.18 ^a	6.4±2.07 ^a	14.2±4.43 ^b
62.5 µg/mL	CHO cells	4.4±2.07	5.2±1.92	7.4±2.07
	L929 Fibroblasts	4.2±1.48	4.8±1.64	5.6±1.94

Note: Data with different letters in superscript in the same row is statistically significant ($P<0.05$). There were significant differences between CHO cells and L929 fibroblasts for any substance used.

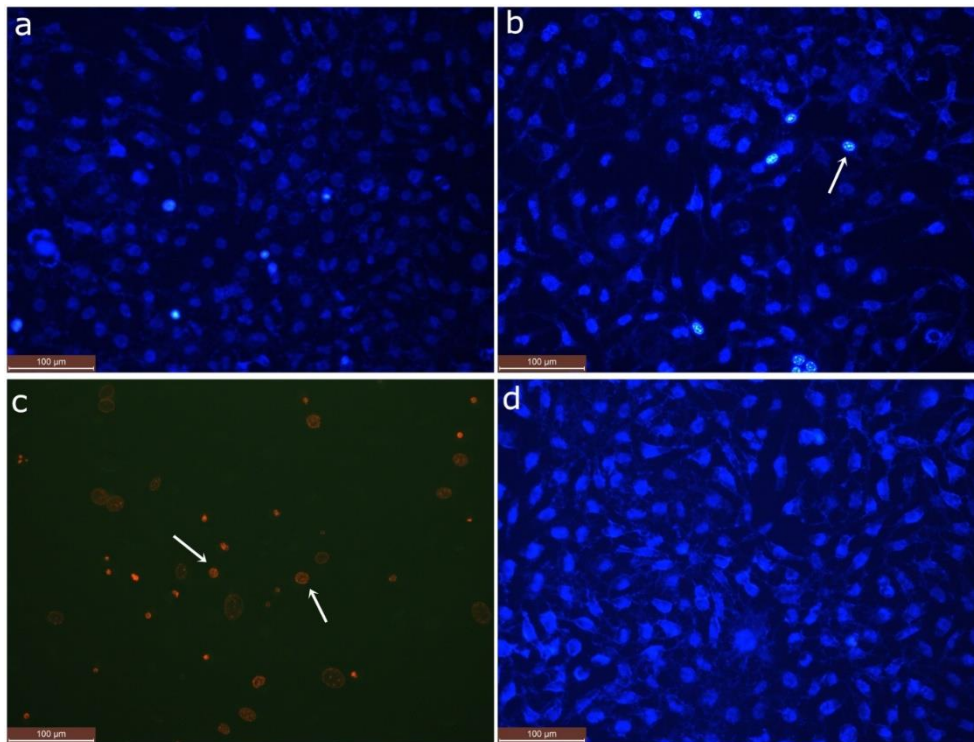


Figure 1. Apoptosis and necrosis in L929 fibroblasts. a) Apoptotic figures following exposure to cyanuric acid at a concentration of 250 µg/mL. b) Apoptotic figures (arrows) following exposure to melamine at a concentration of 125 µg/mL. c) Necrotic figures (arrows) following exposure to the mixture of melamine and cyanuric acid at a concentration of 125 µg/mL. d) Absence of apoptotic figures in untreated cells.

In fluorescence microscopy, the DAPI filter was used to observe Hoechst 33342 staining in a, b, and d while the FITC filter was used to observe propidium iodide staining in c.

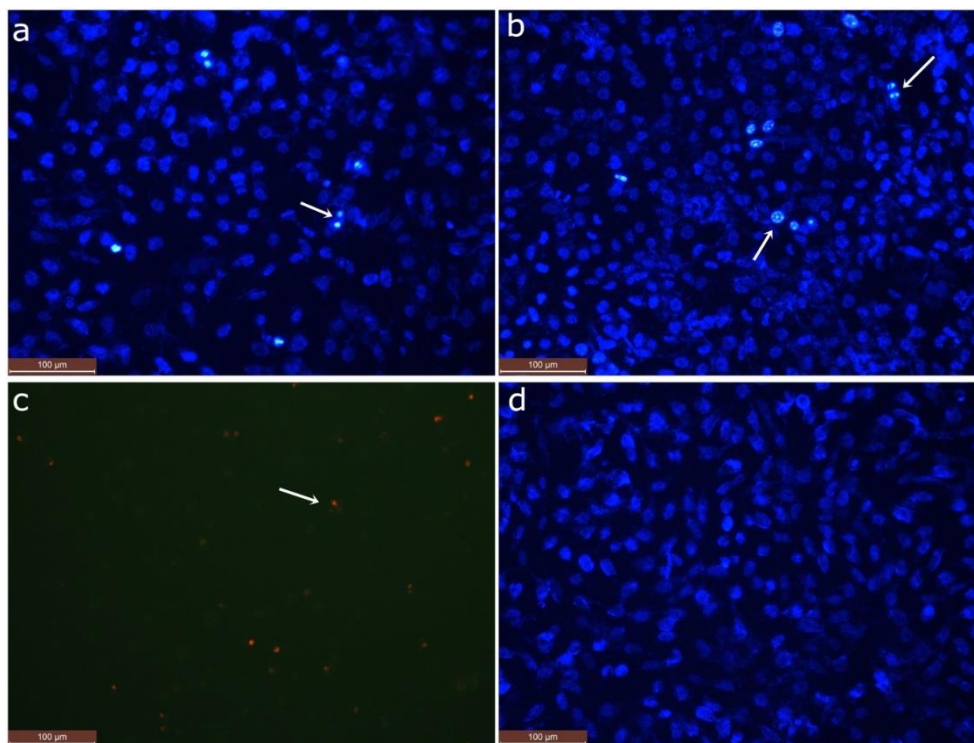


Figure 2. Apoptosis and necrosis in CHO cells. a) Apoptotic figures following exposure to cyanuric acid at a concentration of 250 µg/mL. b) Apoptotic figures (arrows) following exposure to melamine at a concentration of 125 µg/mL. c) Necrotic figures (arrows) following exposure to the mixture of melamine and cyanuric acid at a concentration of 125 µg/mL. d) Absence of apoptotic figures in untreated cells.

In fluorescence microscopy, the DAPI filter was used to observe Hoechst 33342 staining in a, b, and d while the FITC filter was used to observe propidium iodide staining in c.

Discussion and Conclusion

Living organisms are exposed to so many different natural or synthetic materials in various ways such as skin exposure, oral ingestion. In this regard, it is so important to characterize these substances for their biological activities as well as potential toxicities with regard to their chemical nature. *In vitro* cytotoxicity is the first step to reveal the presence or absence of a direct toxic effect at the cellular level. It is also used to illuminate the mechanism of cell death and related pathways. There are various cytotoxicity test including MTT, XTT, neutral red, agar diffusion, and lactate dehydrogenase. In the present study, we preferred MTT test to assess cytotoxicity on L929 fibroblasts and CHO resulting from exposure to melamine, cyanuric acid, and their combination. The MTT test is a colorimetric test and widely used to evaluate cell viability. The MTT test is also called the succinate dehydrogenase activity test because the enzymatic reduction of MTT to MTT-formazan is catalyzed by mitochondrial succinate dehydrogenase in live cells. The cytotoxicity data obtained from the MTT test was supported by the apoptotic and necrotic index data to analyze the pattern of cell death. A double staining method of Hoechst (33342) and propidium iodide (PI) is frequently used to reveal apoptosis and necrosis in cell culture (32). The prominent difference between apoptotic and necrotic cell nuclei is that DNA becomes condensed during apoptosis, but such a condensation process does not occur during necrosis. Nuclear condensation can be revealed by dyes such as Hoechst 33342 those effluxes across the cell membrane and binds to DNA. Unlike Hoechst (33342), PI does not pass across the intact cell membrane. Thus, PI stains DNA and RNA in cells with impaired cell membrane integrity and reveal secondary necrosis. In the present study, we depicted the presence of cell death not only through apoptotic by also necrotic path due to cytotoxicity induced with either melamine, or cyanuric acid or their combinations.

In vitro studies are very important to determine the cytotoxic potential of the substances. Sun et al. (30) concluded that melamine caused cytotoxicity *in vitro* on the rat NRK-52E and human 293T kidney cells with IC_{50} values of 1.89 mg/mL and 2.07 mg/mL, respectively. In Radko et al. (25) study melamine exerted cytotoxicity on the rat FaO hepatoma cells and the rat skeletal L6 cells after 48 hours of exposure with EC_{50} values of 6.4 ± 0.62 mM, and 8.2 ± 1.51 mM, respectively. In the present study, melamine and cyanuric acid caused cytotoxicity in CHO and L929 fibroblasts in a dose-dependent manner. Almost fifty percent of cells died at a concentration of $1000 \mu\text{g/ml}$ following the application of melamine or cyanuric acid alone. Through a rat study as well as an *in vitro* study on the rat NRK-52E and human 293T kidney cells, Sun et al. (30) also concluded that melamine is more toxic than

cyanuric acid. A similar conclusion was made by Choi et al. (8) on ACHN human renal adenocarcinoma cell line and the MDCK canine kidney epithelial cell line.

Although melamine toxicity with the presence or absence of cyanuric acid has been one of public interest, the number of related studies is limited. Some *in vitro* cytotoxicity studies have been conducted. As it has been considered that the kidney is the major target organ in melamine and cyanuric acid, the kidney epithelial cells such as NRK-52E and 293T cell lines were studied *in vitro* (16, 30, 41). It has been revealed that the kidney is not the only target organ for melamine toxicity (37). Thus, some other cell lines including rat hepatoma cells, rat skeletal cells (25), colon adenocarcinoma cells (31), and spleen lymphocytes (37) were investigated for melamine cytotoxicity. In this study, we used two different well-characterized cell lines, CHO and L929. The CHO cell is of epithelial origin and derived from Chinese hamster Ovary (4) while L929 fibroblast is of mouse origin and routinely used in cytotoxicity studies (27). In the present study, CHO cells seem to be more sensitive to melamine with or without cyanuric acid. This difference was often significant in melamine exposed to cells. Such a difference should be further investigated by means of recovery potentials and internal pathways of cells including repair mechanism, scavenging system. The difference in cytotoxicity profile has also been reported by Choi et al. (8) on ACHN human renal adenocarcinoma cells and MDCK canine kidney epithelial cell lines. They found ACHN human renal adenocarcinoma cells were sensitive to melamine, cyanuric acid and their combination than MDCK canine kidney epithelial cell lines. In support of the previous studies, the present study found that melamine seems to be more toxic than cyanuric acid, which is more prominent in CHO cells. Furthermore, both CHO cells and L929 fibroblasts were more sensitive to the mixture of melamine and cyanuric acid than melamine or cyanuric acid alone. This finding was also reported in previous studies. Yin et al. (37) reported that the administration of the mixture of melamine and cyanuric acid caused a declining tendency in the stimulation index of spleen lymphocyte. In another study by Sun et al. (30) concluded that melamine and cyanuric acid results in the formation of a melamine cyanuric acid complex, the toxicity of which is greater than the toxicity of melamine or cyanuric acid alone. In an animal study, Choi et al. (8) observed the absence of adverse effects in renal function in rats treated either with melamine alone or cyanuric acid alone while but nephrotoxicity related findings were present in rats with the mixture of melamine and cyanuric acid.

There have been some studies related to the mechanism of melamine cytotoxicity. Yiu et al. (41)

proposed a mechanism of melamine cytotoxicity in the renal epithelial cell line, LLC-PK1 in which melamine activates Ca²-sensing receptors, which in turn causes a sustained Ca² entry in the renal epithelial cell line, LLC-PK1. Activation of Ca²-sensing receptors also causes a rise in [Ca²]_i that results in increased ROS production. Elevated [Ca²]_i and ROS production cause an increase in apoptotic and necrotic cell death. Yin et al. (37) reported that cell death in spleen lymphocytes as a consequence of exposure to melamine with or without cyanuric acid occurs through an early apoptotic pathway or late apoptotic/necrotic pathways. Guo et al. (13) concluded that melamine increased apoptotic and necrotic rates in the NRK-52e cells in a dose-dependent manner and melamine causes apoptosis in the NRK-52e cells resulting from excessive intracellular ROS production and the activation of p38 MAPK pathway. Han et al. (14) concluded that melamine causes cytotoxicity in differentiated PC12 Cells through apoptosis rather than necrosis. This finding is contradictory to the present study that found cell death due to exposure to melamine, cyanuric acid or their mixture occurs mainly through necrosis. The present study found that cell death thorough apoptosis in CHO cells and L929 fibroblasts following exposure to melamine, cyanuric acid or their mixture is limited. However, the necrotic cell death seems more prominent for both cells. This controversy should be further investigated through other methods and assays.

In conclusion; melamine, cyanuric acid and their mixture cause cytotoxicity in CHO cells and L929 fibroblasts in dose dependent manner. Cell death occurred through both apoptosis and mainly necrosis. Both cell types were more sensitive to the mixture of melamine and cyanuric acid compared to their use alone. When compared to the cells used, CHO cells were more sensitive than L929 fibroblasts to melamine, cyanuric acid and their mixture. As a result, melamine, cyanuric acid and their combination cause cytotoxicity in CHO cells and L929 fibroblasts. Further studies should be conducted in different cell lines. These studies should also aim to reveal the mechanism of cytotoxicity and related pathways.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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