

Original Article

In vitro toxicity of melamine against *Tetrahymena pyriformis* cells

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This study assessed the toxicity of melamine against the unicellular eukaryotic system of *Tetrahymena (T.) pyriformis* exposed to 0, 0.05, 0.25, 0.5, 2.5, and 5 mg/mL of melamine. Cell growth curves of different cultures, the half maximum inhibition concentration (IC₅₀) value of melamine, and morphological changes in cells were obtained via optical and transmission electron microscopic observation. The effects of eleven melamine concentrations, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mg/mL, on protein expression levels of *T. pyriformis* were examined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The results showed an obvious inhibitory effect of melamine on the growth of eukaryotic cells. Cell growth dynamics indicated that the IC₅₀ value of melamine on *T. pyriformis* was 0.82 mg/mL. The cellular morphology was also affected in a concentration-dependent manner, with characteristics of atrophy or cell damage developing in the presence of melamine. The relative contents of the top four main proteins corresponding to peak mass-to-charge ratios (*m/z*) of 4466, *m/z* 6455, *m/z* 6514, and *m/z* 7772 in the MALDI-TOF-MS spectra were all found to be closely correlated with the melamine concentrations. In conclusion, exposure of eukaryotic cells to melamine could inhibit cell growth, cause changes in cytomorphology and even disturb the expression of proteins in a concentration-dependent manner. The described method of examining four sensitive proteins affected by melamine was also proposed to be used in a preliminary study to identify protein biomarkers in *T. pyriformis*.

Keywords: cytotoxicity, MALDI-TOF-MS, melamine, protein biomarker, *T. pyriformis*

Introduction

Melamine, 1, 3, 5-triazine-2, 4, 6-triamine (C₃H₆N₆), is a

common ingredient in fertilizers and a raw material used in the production of plastic products and melamine resin [12]. Since it has a high nitrogen content by mass, it is also added to cattle foodstuffs as a non-protein nitrogen source. Although Newton and Utely [19] pointed out that the hydrolysis of melamine in cattle was incomplete and that it could not be absorbed by ruminants, melamine has been illegally added to foodstuffs to falsely elevate the protein contents determined in assays. Additionally, pet food ingredients contaminated with melamine resulted in a major outbreak of renal disease and associated deaths in cats and dogs in the United States in 2007 [10]. In another incident, infant formula milk powders tainted with melamine caused the death of at least six infants and the hospitalization of 290,000 infants receiving therapy for kidney calculi or renal failure in China in 2008 [9]. The increasing public anxiety regarding food contamination by this potentially toxic chemical substance has made its toxicity the focus of recent investigations by toxicologists and biologists.

After the United States Environmental Protection Agency proposed removal of the “tolerance” expression from the usage guide of melamine, a test method for analyzing melamine in animal tissues was established [2]. At that time, melamine was reported to have an oral LD₅₀ of > 3,000 mg/kg based on rat data and a dermal LD₅₀ of > 1,000 mg/kg for rabbits [3]. Several physiological functions were also found to be disturbed by melamine and/or its derivatives, such as nephrolithiasis, chronic kidney inflammation and bladder carcinoma [6,13,21]. However, there is little information available regarding the use of changes in cellular proteins to assess the toxic effects of melamine on eukaryotic cells.

In the studies reported here, an attempt was made to investigate the side effects of melamine on cells and proteins. The toxicity of melamine has been evaluated using the widely-used single cell system of *Tetrahymena (T.) pyriformis*, which is a ciliated unicellular protozoan that has nutritional requirements, subcellular organelles and biochemical pathways similar to those of mammalian cells [14,16]. In addition, *T. pyriformis* cells have been reported to have sensitivities to toxicants, and this behavior

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is similar to human cell cultures [5]. *T. pyriformis* can be easily cultivated under ambient conditions, and is characterized by a short generation time (approximately 4 to 5 h, but variable under different culture conditions), which allows accumulated toxic impacts of test substances to be studied through several generations in a short period [24]. However, previous studies using *T. pyriformis* to create a bioassay model for investigation of the potential toxicity of many substances have only focused on changes in its physiological state such as locomotory capacity, population growth kinetics, and morphology [4,7,18]. While these analyses have yielded useful information regarding the toxicity of analytes, they have provided limited in-depth analysis of the mechanisms behind the toxicity. We propose that certain proteins could be potential candidates for use as bioindicators of *T. pyriformis* cells that have been exposed to toxicants and subject to melamine-induced stress. In this study, an important analytical technique in proteomics studies, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), was employed as a valid tool for the discovery of biomarkers in the analysis of proteins [25]. This method allows determination of femtomolar amounts of peptide mixtures in a short time [8, 11,26]. Although MALDI-TOF-MS technology is limited in its low reproducibility when used to identify biomarkers in the intact entire pool of cellular proteins, it may be possible to use this method to screen a multitude of proteins in a single spectrum.

This study was conducted to determine if melamine has *in vitro* toxic effects on eukaryotic cells that can be observed based on cellular population growth dynamics and cytomorphology, and measured based on the cellular protein expression level. We discussed the results from the cellular level based on the half maximal inhibition concentration (IC₅₀) and cytomorphology using inverted optical microscopy and transmission electron microscopy (TEM). We then proposed a method of identifying protein biomarkers using MALDI-TOF-MS that overcame the low reproducibility of the technique to some extent. Finally, an assessment of the toxicity of melamine toward eukaryotic cells was conducted *in vitro*.

Materials and Methods

Chemicals and reagents

Analytically pure melamine (C₃H₆N₆), sucrose, D-glucose, NaCl, Na₂HPO₄ · 2H₂O, KH₂PO₄ and KCl were obtained from the Beijing Chemical Reagent Co. (China). Tryptone and yeast extract were purchased from Oxoid (UK). Analytically pure formaldehyde solution was obtained from the Shantou Xilong Chemical Factory (China). Anhydrous ethanol and HPLC grade acetone was purchased from Sinopharm Chemical Reagent (China).

α-cyano-4-hydroxycinnamic acid (CHCA), trifluoroacetic acid (TFA) and formic acid solution were obtained from Sigma-Aldrich (USA). HPLC grade Acetonitrile was obtained from SK Chemicals (Korea). Osmic acid, HPLC grade acetone, uranyl acetate dehydrate puriss. p.a., lead (II) citrate tridhydrate, 25% (wt.) glutaraldehyde solution, and a ProteoMass Protein MALDI-MS calibration kit were obtained from Sigma-Aldrich (USA). Epoxy resin Spurr was purchased from Electron Microscopy Science (USA). Milli-Q Academic ultrapure water (Millipore, USA) was used throughout the experiment. In addition, 0.1 mol/L phosphate buffer solution (pH 7.4) was prepared by adding 80 g NaCl, 11.5 g Na₂HPO₄ · 2H₂O, 2 g KH₂PO₄, and 2 g KCl to 1 L of double distilled water.

Cell culture

T. pyriformis was initially grown as a pure culture at 28°C in basic liquid culture medium containing 1% (w/v) tryptone, 0.1% (w/v) yeast extract, and 0.2% (w/v) D-glucose without shaking for 24 h in darkness.

Population growth curve and the IC₅₀ value of melamine

A series of test culture media were prepared by adding 0.001, 0.005, 0.01, 0.05, and 0.1 g of melamine to 20 mL of basic liquid culture medium to give melamine concentrations of 0.05, 0.25, 0.5, 2.5, and 5 mg/mL, respectively. Two replicas of each test culture medium were prepared. In addition, three negative control culture media containing no melamine were prepared. All flasks were autoclaved at 103 kPa and 121°C for 20 min.

All procedures were conducted using the upper layer of the culture solution. Briefly, 200 μL of the original *T. pyriformis* 24 h culture solution was added to each flask (Time T₀). All cell cultures were then incubated at 28°C in the dark without shaking. Cell counts were conducted microscopically by using an Olympus CX21 microscope (Japan) and a hemocytometer. Each time, 192 μL of cell culture solution was mixed with 8 μL of formaldehyde solution to fix the cells. At time T₀, the initial cell concentration (D₀) of each cell culture was 1 × 10⁴ cells/mL. Cell counts were then conducted every 4 h successively for 52 h.

A plot of log (D_x/D₀) against incubation time (h) yielded linear equations that were used to evaluate the generation times of the control cell culture (GTC) and of each test cell culture (GTT). When the cell concentration doubled, i.e. D_x/D₀ = 2 and log (D_x/D₀) = 0.301, the GTCs and GTTs were calculated from these equations. Consequently, the relative generation time (RGT) was calculated using the ratio RGT (%) = [(GTT - GTC) / GTC] × 100. Finally, a plot of RGT (%) versus the melamine concentrations was presented as a linear regression equation, from which the IC₅₀ value was determined when the RGT (%) was 50 [7].

The growth dynamics of the populations were evaluated by plotting the incubation time versus the D_x/D_0 ratios, where D_x and D_0 were the cell concentration at time $T_0 + x$ h and time T_0 , respectively. The populations were characterized by their generation time, which is the time required for the D_x/D_0 ratio to increase from one to two [7]. The relative toxicity of the melamine is generally quantified by determining the IC_{50} , which is the concentration required to induce a 50% increase in RGT (i.e. GTT is prolonged to 1.5 GTC). If the toxicity of melamine is concentration-dependent, the RGT values could be calculated by monadic linear regression analysis. Data are fitted to the following model: $RGT(\%) = aX + b$ [7], where X represents the IC_{50} value of melamine when RGT (%) is 50.

The cellular morphology at different stages of growth was observed by using an inverted microscope (IX71; Olympus, Japan).

Transmission electron microscopic observation

After 48 h of incubation, the flask containing the cell culture was shaken slightly to give a cell suspension solution. After being allowed to stand for 10 min, 5 mL of the cell suspension were removed from near the surface layer of the culture medium using a pipette and placed into a centrifuge tube. The cells were then harvested by centrifugation at 7,000 rpm for 2 min at room temperature. The supernatant was discarded immediately after the centrifugation, after which 450 μ L of ultrapure water was added to the tube to disperse the aggregated cells. Next, 50 μ L of 25% (w/v) glutaraldehyde solution was added to the solution, to fix the samples, and the mixture was stored at 4°C overnight. The mixture was then centrifuged at 7,000 rpm for 2 min at 4°C, after which the supernatant was immediately discarded. Next, 500 μ L of 0.1 mol/L phosphate buffer solution (pH 7.4) was added to the centrifuge tube to rinse the cells three times. The sample was then centrifuged at 7,000 rpm for 2 min at 4°C, after which the supernatant was discarded and 500 μ L of 1% osmic acid solution was added. The mixture was then centrifuged at 7,000 rpm for 2 min at 4°C, after which the supernatant was discarded and 500 μ L of 0.1 mol/L phosphate buffer solution (pH 7.4) was added to rinse the precipitates three times. The specimen was then harvested by centrifugation at 7,000 rpm for 2 min at 4°C. The samples were then dehydrated by gradient elution using 30%, 50%, 70%, 80%, 90% and 100% (v/v) acetone in sequence. For each concentration of acetone, 500 μ L of the acetone solution was added to the precipitates and then removed after 5 min. Next, the samples were subjected to the epoxy resin coating technique. Briefly, the specimen and the liquid resin with sclerosing agent were mixed thoroughly, allowing the specimen to become embedded in the liquid mixture. The liquid resin was then allowed to

harden into a transparent solid. Finally, the naturally solidified resin containing the TEM specimen was divided into ultrathin sections using the ultramicrotome (Ultracut UC6i; Leica, Germany), and then placed on the sample stage of the TEM (JEM-1230; JEOL, Japan) for observation.

MALDI-TOF-MS analysis

A new series of culture media were prepared by adding 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1 g of melamine to 20 mL of refresh basic liquid culture medium to give final concentrations of 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mg/mL, respectively. All flasks were then autoclaved at 103 kPa and 121°C for 20 min, after which they were allowed to cool. Next, 200 μ L of original *T. pyriformis* culture solution was added to each flask and incubated at 28°C in the dark without shaking.

After 48 h of incubation, 10 mL of the upper layer of the cell culture solution was withdrawn from each flask and centrifuged at 7,000 rpm for 10 min at 4°C. The supernatant was then discarded, after which 30 μ L of water plus 90 μ L of alcohol were added. The samples were then centrifuged again at 10,000 rpm for 2 min at 4°C, after which the supernatant was discarded and 50 μ L of 70% formic acid solution plus 50 μ L of acetonitrile were added to the precipitate. The samples were then immediately placed at -70°C for 30 min, after which they were subject to 10 min of ultrasonication and then placed at -70°C again for 30 min. This freezing and thawing procedure was conducted three times.

Following centrifugation at 10,000 rpm for 2 min at 4°C, 0.5 μ L of the supernatant was spotted directly onto one well of the stainless MALDI plate and allowed to air dry. Next, 0.5 μ L of the matrix saturated solution was immediately added to the dried sample spot. Fresh matrix solution containing 10 mg/mL CHCA in a 50% acetonitrile/0.1% TFA water solution was prepared daily.

All MS experiments were conducted using a 4,700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA) equipped with an Nd: YAG laser (355 nm). Mass spectra were obtained in the positive ion linear mode with a 740 ns delay time at an acceleration voltage of 20 kV. The bin size was 10 ns, and the input bandwidth was 25 MHz. Each mass spectrum was the sum of 60 sub-spectra and 25 shots were averaged automatically per sub-spectrum. The instrument was externally calibrated using a standard bovine insulin solution.

Results

Effects of melamine on the *T. pyriformis* population growth rate

Melamine was found to have a concentration-dependent inhibitory effect on *T. pyriformis*. Specifically, as the

melamine concentration increased, the growth of *T. pyriformis* was inhibited and its generation time was prolonged accordingly.

Time courses of the cell concentration (cells/mL) are shown in Fig. 1. The presence of 0.05 mg/mL of melamine in the culture medium did not have a visible effect on cell growth, whereas increasing amounts of melamine in the culture medium resulted in greater inhibition of cell growth. After 20 h of incubation, the concentrations of different test cultures began to show noticeable differences, which were used to correlate the cell concentration with melamine concentrations.

A logarithmic plot of (D_x/D_0) against incubation time was used to evaluate the GTCs and GTTs by linear equations (Table 1). Based on the control culture ($y = 0.1144 + 0.0325x$), the GTC was determined to be 5.7 h in this study, while the test cultures had longer generation times. The presence of melamine caused a concentration-dependent decrease in population growth.

RGT (%) was then calculated from the GTC and GTTs data (Table 1). A plot of RGT (%) versus the concentrations provided the following equation, $y = -13.91 + 79.184x$, with a relation coefficient of 0.9645. When the RGT (%) was 50, the IC_{50} value of melamine was determined to be 0.82 mg/mL.

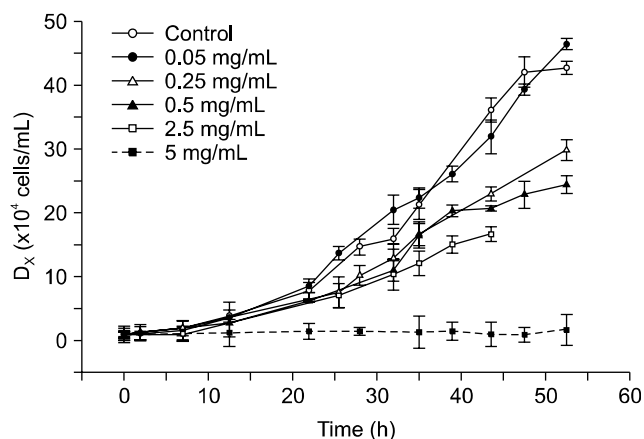


Fig. 1. Growth dynamics of *Tetrahymena* (*T.*) *pyriformis* cells exposed to different concentrations of melamine. The presented cell concentration (D_x) was the mean of three replicates. Although the D_x of different cell cultures increased, cell growth was obviously inhibited in the presence of melamine. At various time spots, the D_x of each test culture was compared with that of the control culture using the Paired-Samples *t* test in the SPSS 16.0 software. The results showed that there were no significant differences between the test culture containing 0.05 mg/mL of melamine and the control group at any time point ($p > 0.05$). After 26 h of exposure to 0.25, 0.5, 2.5 and 5 mg/mL of melamine, the test cultures differed significantly from the control culture ($p < 0.05$).

Effects of melamine on the *T. pyriformis* cell morphology

Optical microscopy revealed a normal pear-like shape of *T. pyriformis* (Figs. 2A and 3A). Under the experimental conditions, the *T. pyriformis* cell shape was not visibly influenced by the presence of 0.05 mg/mL melamine (Fig. 2B), whereas cells exposed to 0.25 mg/mL melamine showed characteristics of atrophy or deformation. An obvious decrease in cell number was also observed (Figs. 2C and 3B). *T. pyriformis* cells could not maintain a normal

Table 1. The generation time of the control culture and test cultures of *Tetrahymena pyriformis*

Concentration of melamine (mg/mL)	Linear equations	Relation coefficient	Generation time (h)
0	$y = 0.1144 + 0.0325x$	0.9871	5.7
0.05	$y = 0.0864 + 0.0336x$	0.9811	6.4
0.25	$y = 0.1077 + 0.0291x$	0.9834	6.6
0.5	$y = 0.1040 + 0.0279x$	0.9822	7.1
2.5	$y = -0.0674 + 0.0320x$	0.9841	11.5
5	$y = -0.0115 + 0.0104x$	0.9986	30.0

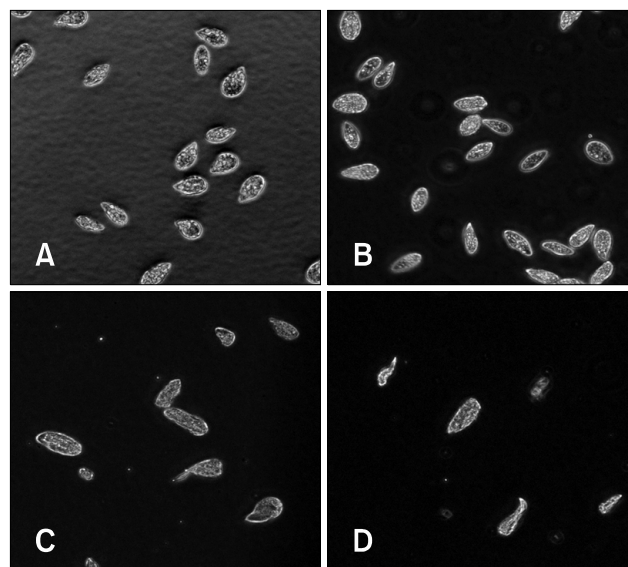


Fig. 2. Inverted microscopic images of melamine-treated *T. pyriformis* cell cultures after 48 h of incubation. (A) Normal pear-like shaped *T. pyriformis* cells in the negative control culture. (B) Cellular shape and cell number were not obviously influenced by the presence of 0.05 mg/mL of melamine. (C) Cell number decreased evidently and cellular shape became irregular when the melamine concentration was 0.25 mg/mL. Characteristic cell atrophy appeared. (D) Cell number decreased drastically and many cells were damaged when the melamine concentration reached 2.5 mg/mL. $\times 40$.

pear-like shape when the melamine concentration reached 2.5 mg/mL (Fig. 3C), and many of them were damaged (Fig. 2D).

Electron microscopic imaging of *T. pyriformis* revealed alterations of the intracellular structure. When the cell was in a normal condition, its plasma membrane covered the entire organism and was smooth and integrated. In the cell, the cytoplasm was clear and homogeneous, and large mucocysts that were oblong in shape and contained mucoid material appeared to play a role in the endocytic uptake of nutrients (Fig. 4A). However, when *T. pyriformis*

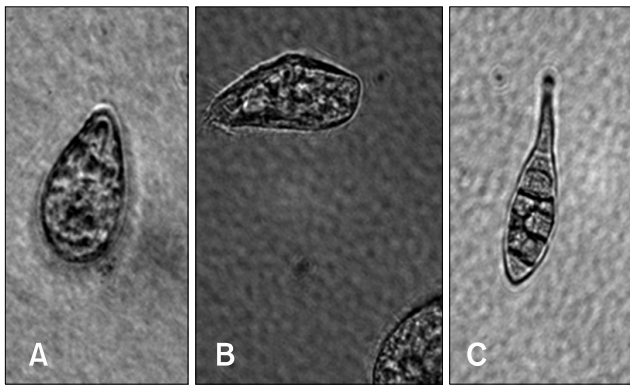


Fig. 3. Inverted microscopic images of melamine-treated *T. pyriformis* cells after 48 h of incubation. (A) A pear-like shaped normal *T. pyriformis* cell in the negative control culture. The cell membrane was integrated and its cytoplasm was clear and homogeneous. (B) Characteristic cell atrophy in the presence of 0.25 mg/mL of melamine. The cell membrane was damaged, and cytoplasm was not abundant. (C) *T. pyriformis* cells were severely atrophic when the melamine concentration was 2.5 mg/mL. $\times 200$.

cells were grown in culture medium containing 0.5 mg/mL of melamine, the plasma membrane became folded, and the number of mucocysts decreased dramatically (Fig. 4B). In addition, *T. pyriformis* had only one spherical, polyploidy macronucleus, about 10 μm in diameter, which was covered by a nuclear membrane to form a closed region (Fig. 4A). Furthermore, the chromatin granules, which are composed of DNA and proteins, were visible as small dark particles in the macronucleus region (Fig. 4). In the presence of 0.5 mg/mL of melamine, the nucleoli of *T. pyriformis* cells aggregated to form denser and larger fusion bodies associated with thread-like elements (Fig. 4B). When the concentration of melamine in the culture medium reached 2.5 mg/mL, the nuclear membrane was damaged and the macronucleus region of the configuration disappeared from the *T. pyriformis* cells (Fig. 4C).

Effects of melamine on the *T. pyriformis* protein expression level

After 48 h of incubation, *T. pyriformis* was in the middle of its logarithmic growth phase (Fig. 1). The control culture and test cultures were analyzed simultaneously by MALDI-TOF-MS. When the concentrations of melamine in the culture medium changed, the MALDI-TOF-MS spectra of *T. pyriformis* differed accordingly (Figs. 5A-C), indicating that melamine influenced the protein expression of *T. pyriformis* cells.

Melamine showed different effects on different proteins (Fig. 6). Initially, the relative intensity of m/z 4466 increased in response to the increasing concentrations of melamine up to 3 mg/mL, after which it decreased. This pattern of alterations in relative peak intensity was opposite in the case of the peaks at m/z 6455, m/z 6514 and

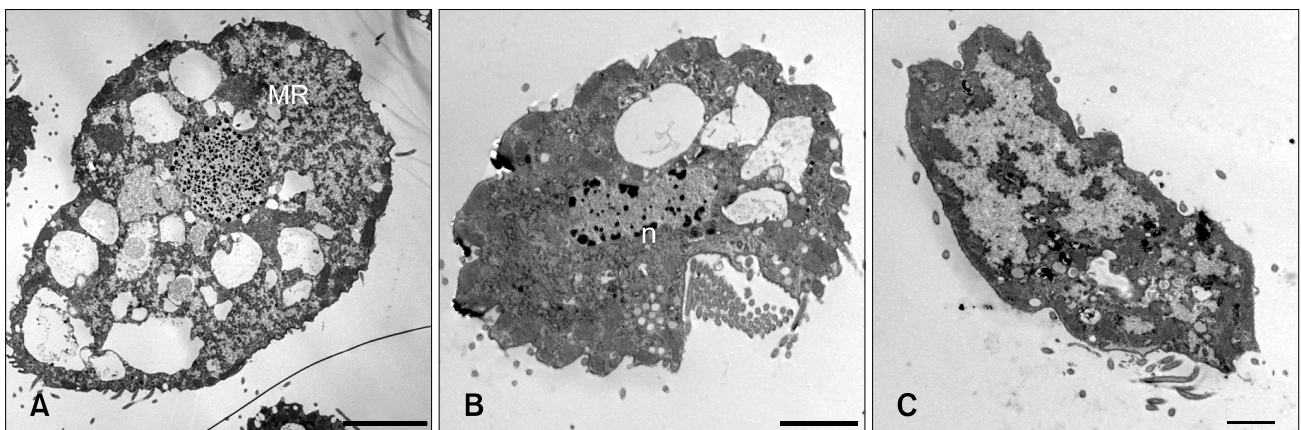


Fig. 4. Transmission electron microscopic images of *T. pyriformis* cells after 48 h of incubation. The concentration of melamine in culture medium A was 0 mg/mL, while it was 0.5 mg/mL in B and 2.5 mg/mL in C. In A, the plasma membrane of *T. pyriformis* cell was smooth and integrated, and the cytoplasm containing many large mucocysts was clear and homogeneous. Normal nucleoli distributed in the macronucleus region are shown in A, while thread-like nucleoli aggregates in the melamine-treated cell are shown in B. In C, the macronucleus region and mucocysts disappeared, and the previously homogeneous cytoplasm showed a character of porosity. MR: macronucleus region, n: nucleolus. Scale bars = 5 μm .

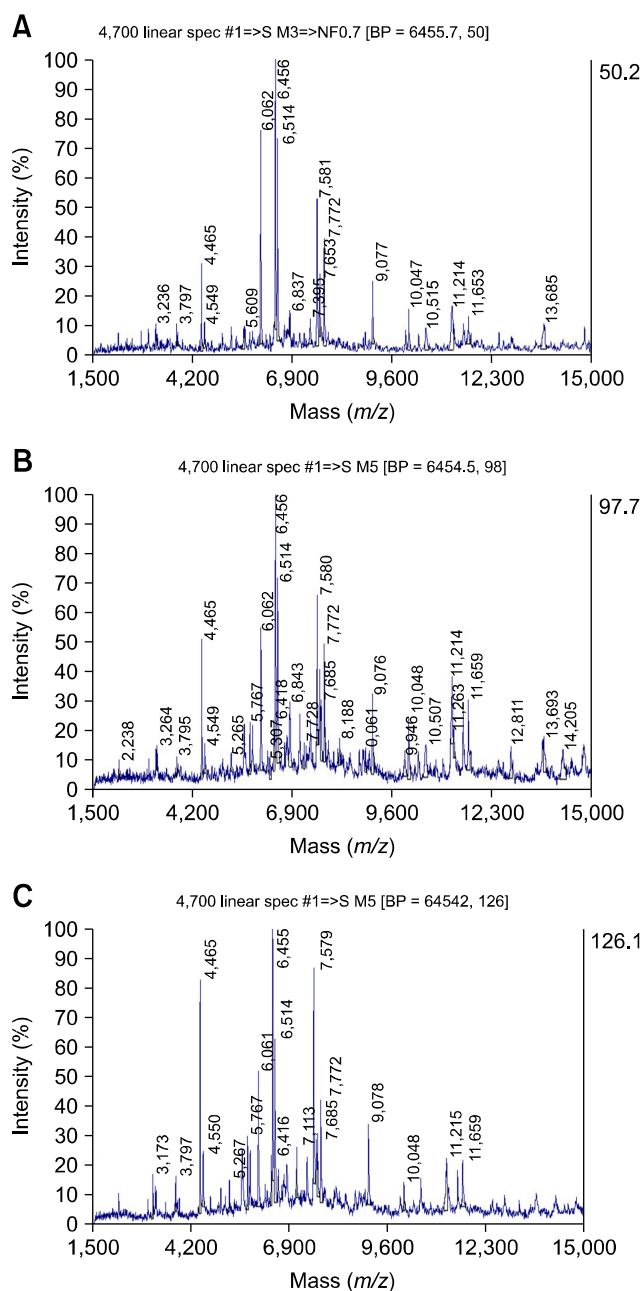


Fig. 5. MALDI-TOF-MS spectra of *T. pyriformis* intact proteins after 48 h of incubation. The concentration of melamine was 0 mg/mL (A), 3.0 mg/mL (B), and 4.5 mg/mL (C). Peaks at m/z 4466, m/z 6455, m/z 6514, and m/z 7772 in the MALDI-TOF-MS spectra corresponded to the four main proteins in *T. pyriformis* cell. Their relative intensities changed in response to gradual increases in the melamine concentrations.

m/z 7772 (Fig. 6). Taken together, these findings indicate that 3 mg/mL of melamine was a joint turning point that caused a reversal in the protein expression levels.

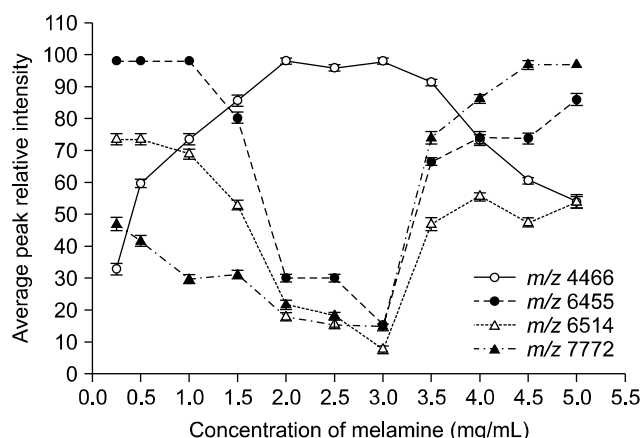


Fig. 6. Alterations in the relative intensities of peaks m/z 4466, m/z 6455, m/z 6514, and m/z 7772 in the MALDI-TOF-MS spectra. The turning point of each of these polygonal lines was 3 mg/mL melamine. Peaks m/z 6455, m/z 6514 and m/z 7772 showed a first down-regulation and then up-regulation of their relative intensities before and after the turning point, whereas peak m/z 4466 showed a first up-regulation and then down-regulation of its relative intensity before and after the turning point.

Discussion

To the best of our knowledge, no other studies have been conducted to examine the toxic effects of melamine in cell cultures, although clinical studies have provided evidence of the toxicity and physiological effects of melamine on animals [15,17,21,22]. Melamine has been reported to have low acute toxicity, with an oral LD_{50} in rats of 3,161 mg/kg [3]. The results of the present study showed that melamine also has a concentration-dependent inhibitory effect on *T. pyriformis* cells *in vitro*, with an IC_{50} value of 0.82 mg/mL, and that it eventually induces cytotoxicity. In the presence of melamine, *T. pyriformis* cells gradually became atrophic and were finally damaged in response to increasing melamine concentrations in culture media. Additionally, electron microscopy revealed alterations in the intracellular structure of *T. pyriformis*. Although low doses of melamine had no visible toxic effect on *T. pyriformis*, prolonged exposure to a low dose of melamine could be harmful to eukaryotic cells. Previous studies have shown that dose- and time-dependent exposure of cells to toxic substances can induce different cellular stress responses [23]. After a long exposure to melamine, accumulation of a low dose of this low toxic chemical substance may also influence the normal function of cells. The results of this study also demonstrate that melamine exerts a concentration-dependent toxic effect on eukaryotic cells that is accumulated over several generations. Thus, these findings demonstrate that melamine cannot be administered as a non-nitrogen source for eukaryotes.

Additionally, the simplicity, sensitivity and rapidity of

this experiment as well as the low culture requirements demonstrate that *T. pyriformis* is an ideal model for *in vitro* cytotoxic bioassay [20,24], and that *T. pyriformis* bioassays can be used for the risk assessment of potentially toxic chemical substances.

In the present study, intact *T. pyriformis* proteins were harvested via repeated freezing and thawing, and proteins from different cell cultures were analyzed simultaneously by MALDI-TOF-MS. The presence of melamine in the test culture media resulted in dramatic deviations in the MALDI-TOF-MS spectra when compared with the negative control culture. Specifically, the relative intensities of the peaks corresponding to different proteins were influenced differently by the melamine. This nonspecific regulatory influence of melamine on protein expression levels indicates that its toxic effect at the physiological level may affect several cellular mechanisms.

Peaks with a high signal-to-noise ratio ($S/N > 5$) and a high reproducibility were selected as representative peaks. Four matched peaks, m/z 4466, m/z 6455, m/z 6514, and m/z 7772 ($m/z \pm 1$), appeared in all of the MALDI-TOF-MS spectra. Moreover, the relative intensities of these peaks were much higher and more sensitive to the influences of melamine than that of any other peaks. Therefore, we assumed that these alterations in protein expression levels might indicate an unhealthy status of cells, such as toxicant-induced cell stress. Accordingly, the identification of bioindicators in a follow-up study may facilitate the monitoring and control of bioreaction processes.

We further assumed that these four proteins may be related to the metabolism of melamine or other toxicants in *T. pyriformis*. Before the concentration of melamine reaches 3 mg/mL, cell growth is only inhibited, indicating that cell metabolism may be interfered with by the presence of melamine and that the deformation of *T. pyriformis* cells may be caused by the irregularity of cellular protein expression levels. When the concentration of melamine increases, the accumulation of melamine in cells exceeds the normal metabolic capability of *T. pyriformis*, and most cells are damaged. Therefore, the alteration trends in the relative contents of these four representative MALDI-TOF-MS peaks differ before and after the turning point. As a result, these up- and/or down-regulated cellular proteins may be useful for indicating cell responses to different concentrations of melamine. However, these four low-weight proteins in *T. pyriformis* have not been reported previously; therefore, their actual functions in cells should also be further studied, and our understanding of the intracellular process pertaining to melamine or other toxicant induced cell stress still needs to be expanded.

In this study, the cytotoxic effects of melamine were evaluated *in vitro* at both the cellular level and the protein level, and proteins sensitive to the presence of melamine

were identified. The results indicated that melamine influences the growth and morphology of eukaryotic cells, and that it affects the cellular protein expression levels. Future studies should focus on the identification of proteins corresponding to the most important discriminating peaks in the MALDI-TOF-MS spectra.

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