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AN INVESTIGATION ON THE DNA BINDING ACTIVITIES OF MELAMINE, CYANURIC ACID AND URIC ACID

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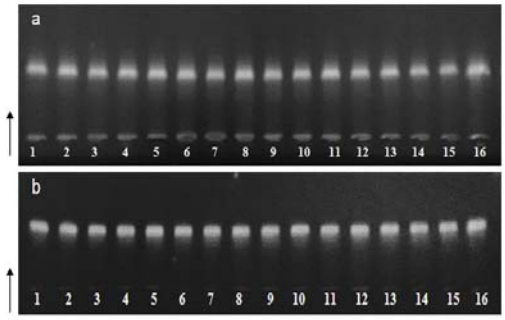
Melamine can be added to various foods such as milk, milk powder, baby food, pet, and livestock feed for cheating purposes due to its high nitrogen content. Regarding its usage in food products, there is a need to investigate its possible interactions with DNA. Thus, this study aimed to investigate the interactions of melamine and its metabolized products, cyanuric acid and uric acid with genomic DNA, isolated from eukaryotic (calf thymus) and prokaryotic (*Staphylococcus aureus*) sources. UV-absorbance spectrophotometry, fluorescence spectrophotometry, and agarose gel electrophoresis techniques were used to evaluate these interactions. The five different concentrations of melamine, cyanuric acid, and uric acid were incubated with fixed DNA concentration and it was determined that the test compounds interacted with the DNA molecules. The data obtained by UV-absorbance and fluorescence spectrophotometry techniques revealed an increase in wave peaks observed with the increasing substance concentration. After the obtained data of the aforementioned techniques were evaluated together, it was concluded that melamine, cyanuric acid, and uric acid bonded to the eukaryotic and prokaryotic genomic DNA materials via groove binding.



INTRODUCTION

Melamine (2,4,6-triamino-1,3,5-triazine, $C_3H_6N_6$) is a chemical compound with a number of industrial uses, including in the production of laminates, glues, kitchenware, adhesives, moulding compounds, coatings, and flame retardants.[1,2] However, due to its high nitrogen content (66.6%), it has been used in some food products such as wheat gluten, rice protein, milk, milk powder, baby food, pet and livestock feeds to provide fake protein height in foods.[3] Serious health problems, even deaths, have been reported in many infants and animals consuming these fraudulent foods that are placed on the market.[4,5] Structural metabolites of melamine, such as cyanuric acid (2,4,6-trihidroksi-1,3,5-triazin, $C_3H_3N_3O_3$) and uric acid (2,6,8-trihidroksipürin, $C_5H_4N_4O_3$) can be added directly to the foods or they can appear as the decomposition products of melamine.[6,7] Also, metabolically produced uric acid can be found in physiological conditions as a result of purine metabolism of the body.[8] It has been determined that melamine cannot be metabolized and has a plasma half-life of about 3 hours.[7] Accordingly, it has been concluded that the acute toxicity of melamine, which is removed from the urinary system in a short time, is low.[9] However, it was also stated that the combination of melamine with cyanuric acid or uric acid induces the increase of nephrotoxicity.[9,10] In the light of recent reports, this would be due to melamine cyanurate crystals

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formed by melamine and cyanuric acid, or urinary tract stones produced by melamine and uric acid. Additionally, the accumulation of formed crystals and stones in the kidney(s) can cause pathological conditions such as tubular blockage and degeneration, and even deaths due to kidney failure.[11,13] Although, the clinical symptoms are associated mainly with kidney, ureter or bladder stone, other potential harmful effects remain uncertain. Hence, it is necessary to determine their interactions with DNA, which plays a critical role in life processes. In recent years, the studies on binding of small molecular compounds such as drugs,[14,15] pesticides,[16] metals and complex compounds[17-19] to DNA has attracted great interest of many researchers. Understanding the DNA binding mechanism of molecules is very useful in predicting the results of molecular interactions in the living body and in designing new or effective drugs.[20,21] Thus, this study aimed to determine the interactions of melamine and its structural metabolites with DNA molecule. The study further aimed to determine whether these effects are different in eukaryotic and prokaryotic DNA. The effects of melamine, cyanuric acid and uric acid on DNA were investigated *in vitro* on eukaryotic calf thymus and prokaryotic *S. aureus* DNA. For this purpose, UV-absorbance spectrophotometry, fluorescence spectrophotometry and agarose gel electrophoresis techniques were applied to evaluate the interactions between the tested compounds and DNA molecules in various concentrations.

RESULTS AND DISCUSSION

UV-Absorbance Spectrophotometry

The results of our study showed that as expected eukaryotic and prokaryotic DNA samples had an absorption peak at 260 nm. Aside from this, melamine, cyanuric acid, and uric acid revealed different absorption peaks (Fig. 1). To investigate the interactions between structural metabolites and DNA, we performed several combinations at different concentrations of melamine, cyanuric acid, and uric acid (Fig. 2). The fact that the graphs obtained by the measurements of calf thymus DNA (ct-DNA) and *S. aureus* DNA were similar. Thus, there was no significant difference among two DNA types. It was also determined that the hyperchromic effect was observed in the graphs due to the intermolecular groove binding, and this was consistent with the literature.[22]

Fluorescence spectrophotometry

DNA and melamine or its structural metabolites (melamine/metabolites) were seen to reveal low fluorescence in optimization trials. Fluorescence spectra of the combinations of DNA+melamine/ metabolites in the presence of SafeView™ DNA dye were measured. Graphs representing the change in fluorescence intensity were created with the data obtained (Fig. 3). When the findings were evaluated together, it was observed that SafeView™ DNA dye, which diffused between DNA base pairs by intercalation, caused an increase in the fluorescence spectra of the DNA molecule. If the interaction of melamine and its metabolites with DNA was in the form of intercalation, the molecules competing with the dye would have caused fluorescence extinction and a decrease in wavelengths and that would have been expected to be in relation with the substance concentration. But the presence of hyperchromic effect in the graphs performed with both ct-DNA and *S. aureus* DNA showed that the intermolecular interaction was not through intercalation. It was observed that there were no significant differences between DNA types, but higher fluorescence spectra were taken in measurements with ct-DNA. In consequence, these results were consistent with our UV-absorbance data and strengthened the opinion that the interaction between molecules are in the form of groove binding and also coincides with the conducted similar studies.[6-23]

Agarose gel electrophoresis

The effects of melamine and its metabolites on genomic DNA band volume and electrophoretic mobility were evaluated. Obtained gel images of the ct-DNA and *S. aureus* DNA are given in Fig. 4.

It was clear that after binding of melamine and metabolites, the intensity of ct-DNA and *S. aureus* DNA bands were almost the same. In the analysis of gel images in the imaging program, pixel measurements of the DNA band intensities showed that as the loading concentration increased, there was a decrease in the volumes of the DNA bands.

As seen in the relevant images (Fig. 5), it was clear that the maximum band volume was obtained in the sample 16 (Fig. 4), which was used as non-interacted control DNA. It was determined that all concentrations of all three compounds combined with DNA caused a decrease in band volumes, and this reduction was more evident in the mixtures with melamine.

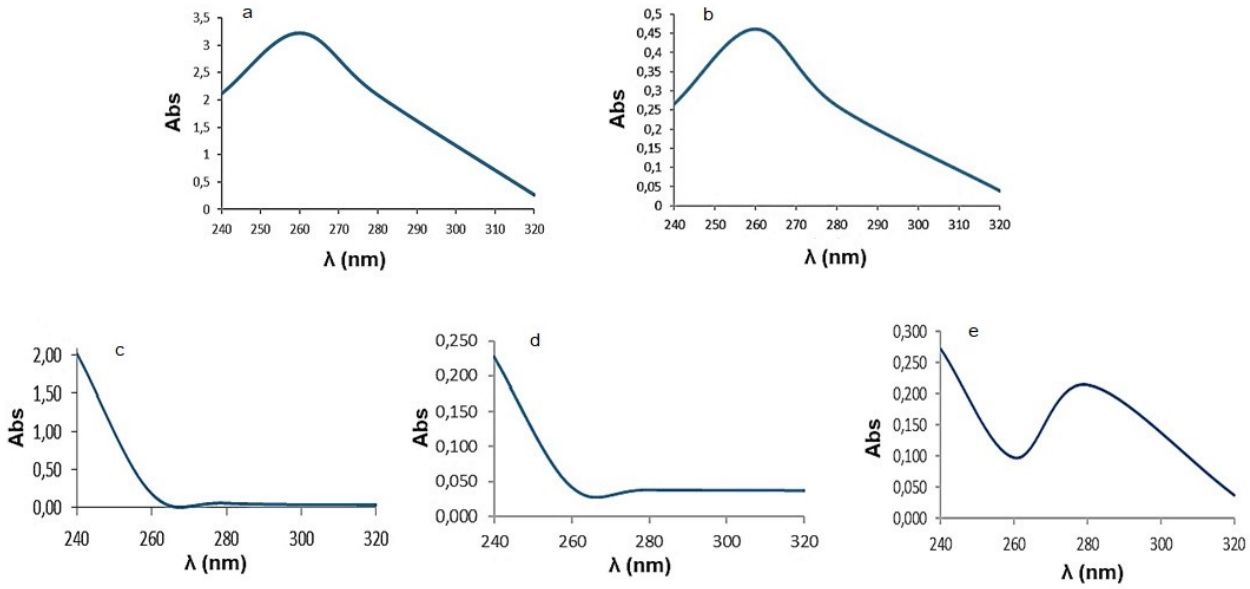

Fig. 1 – UV-absorbance spectra of the tested molecules (a – ct-DNA, b – *S. aureus* DNA, c – Melamine 10 mM, d – Cyanuric acid 15 mM, e – Uric acid 1 mM).

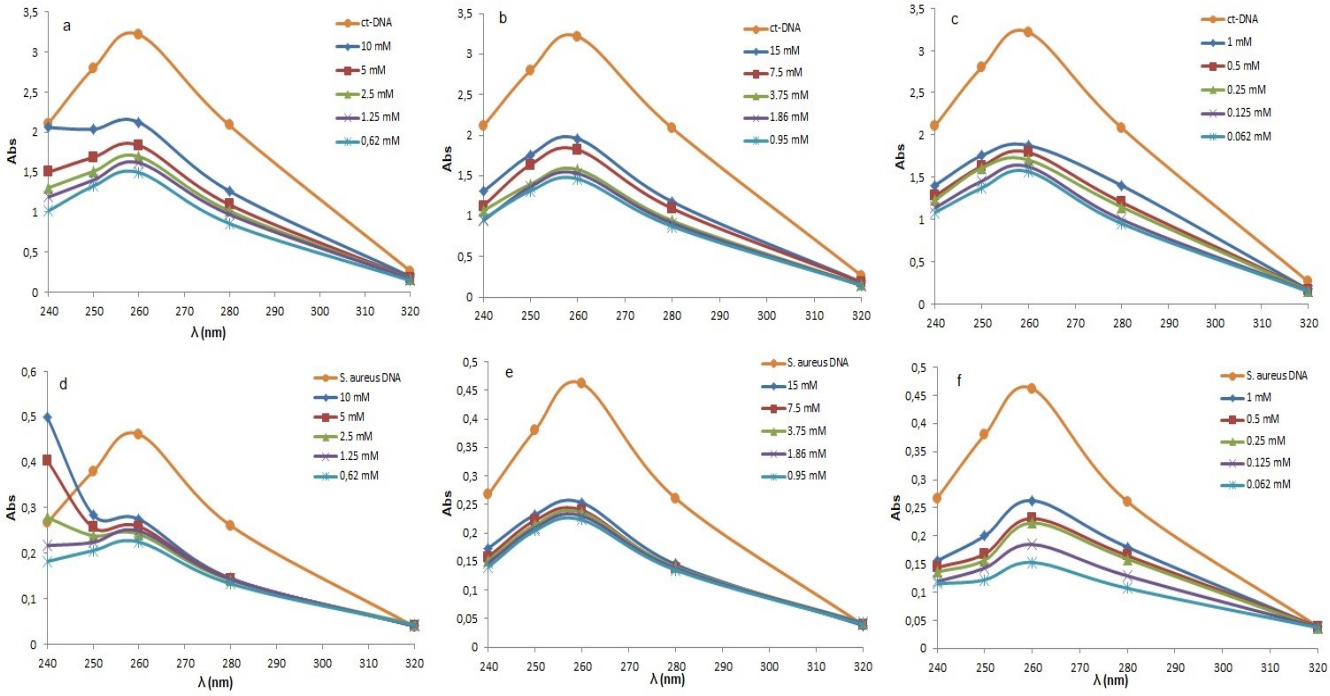

Fig. 2 – UV-absorbance spectra of genomic DNA+melamine/metabolites. (a) ct-DNA+melamine, (b) ct-DNA+cyanuric acid, (c) ct-DNA+uric acid, (d) *S. aureus* DNA+melamine, (e) *S. aureus* DNA+cyanuric acid, and (f) *S. aureus* DNA+uric acid.

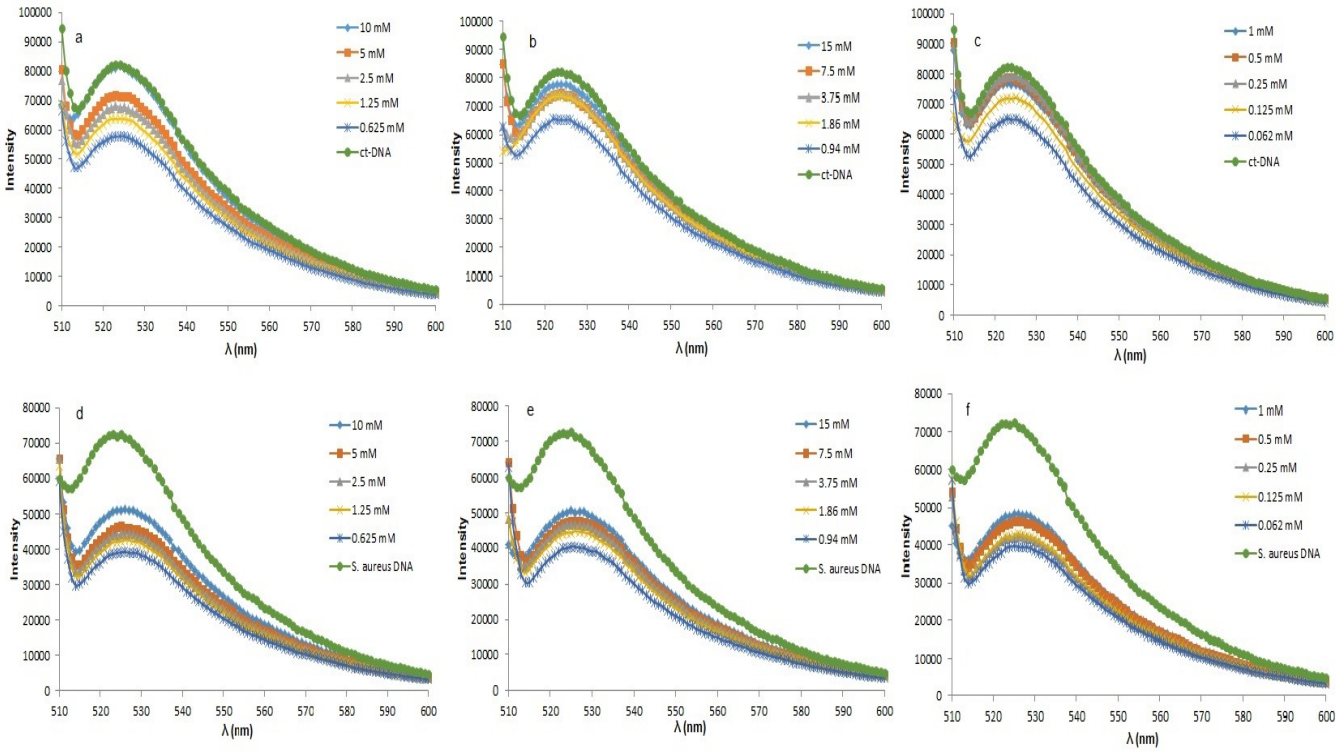

Fig. 3 – Fluorescence spectra of genomic DNA+melamine/metabolites. (a) ct-DNA+melamine, (b) ct-DNA+cyanuric acid, (c) ct-DNA+uric acid, (d) *S. aureus* DNA+melamine, (e) *S. aureus* DNA+cyanuric acid, (f) *S. aureus* DNA+uric acid.

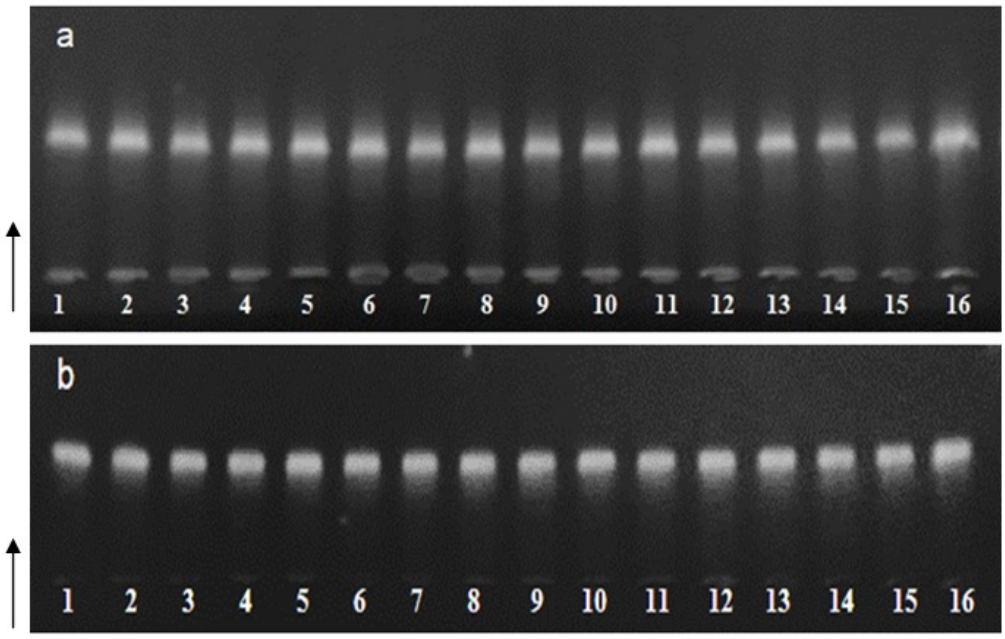

Fig. 4 – DNA+melamine/metabolites agarose gel images: (a) ct-DNA and (b) *S. aureus* DNA. Lanes 1,2,3,4 and 5 exhibit DNA+melamine (0.62, 1.25, 2.50, 5, 10 mM), lanes 6,7,8,9 and 10 show DNA+cyanuric acid (0.95, 1.86, 3.75, 7.50, 15 mM), lanes 11,12,13,14 and 15 indicate DNA+uric acid (0.062, 0.125, 0.25, 0.50, 1 mM), lane 16 is the control DNA. Arrows indicate the direction of DNA migration in agarose gel.

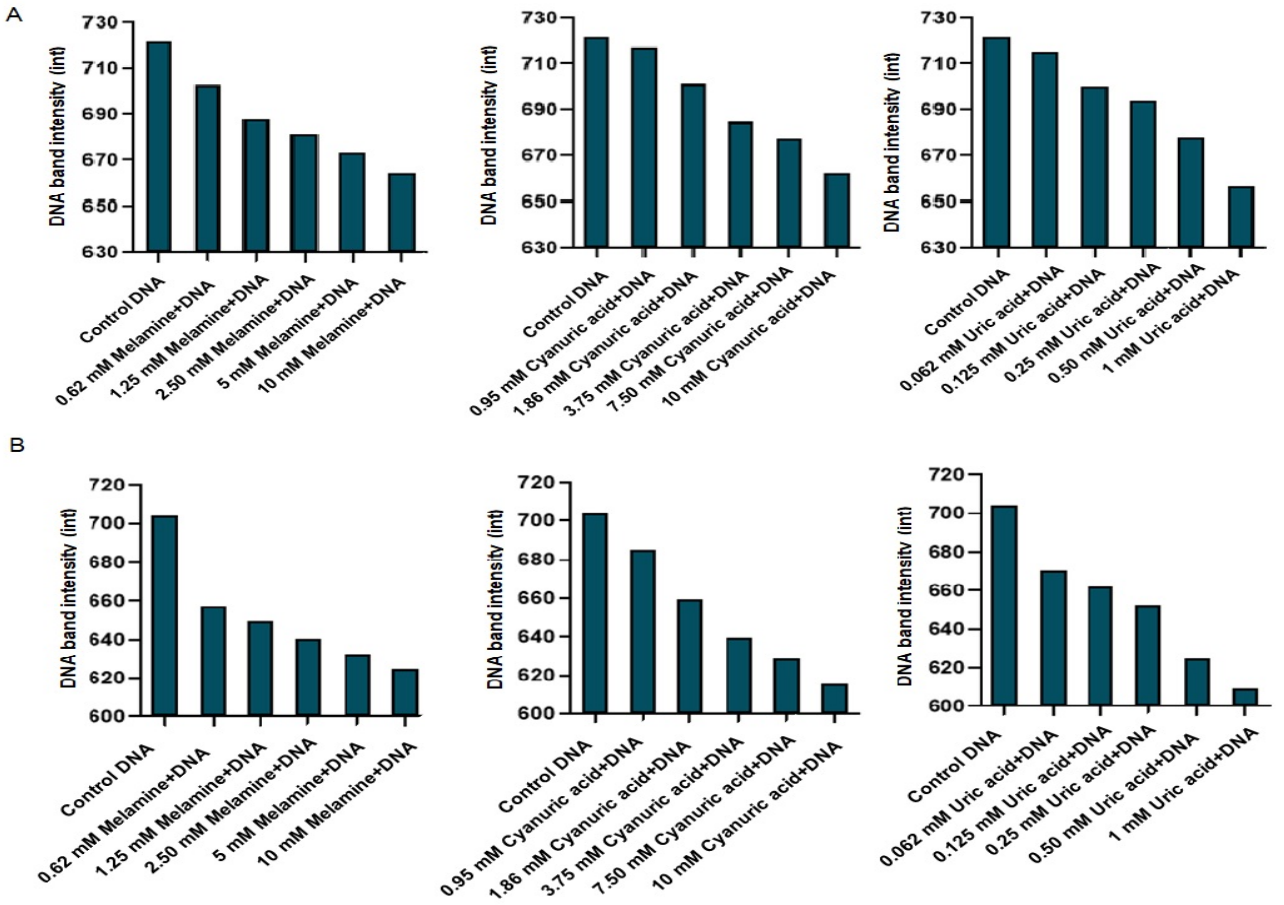

Fig. 5 – DNA band intensities obtained from images of agarose gel electrophoresis. A: Calf thymus genomic DNA+melamine/metabolites, B: *S. aureus* DNA+melamine/metabolites.

EXPERIMENTAL

DNA Isolation

The experimental protocol was approved by the Ethics Committee at Kırıkkale University (Decision no 2019/E9895). Calf thymus tissue was preferred for eukaryotic DNA isolation because of its rich DNA molecules and allows more DNA isolation. Calf thymus tissue was collected from the municipal slaughterhouse of Kırıkkale city localized in Central Anatolia. DNA isolation was performed with the Wizard® Genomic DNA Purification Kit (Promega, A1120, USA). In the evaluation of prokaryotic DNA, *S. aureus* (ATCC 25923) was used, and DNA isolation was carried out as described previously.[24] The quantities and purity (260/280 nm ratio) of extracted DNA samples were determined with a nanodrop spectrophotometer device (Multiskan GO, Thermo, Waltham, USA) and 250 ng/µL concentration was used in DNA binding experiments.

Chemicals

Melamine, cyanuric acid, and uric acid were purchased commercially (Sigma, Burlington, USA). Tris-HCl-NaCl (pH: 7.4) and Tris (hydroxyl-methyl) aminomethane (Merck, Darmstadt, Germany), NaCl (Carlo Erba Reagent®, Val de Reuil, France) and HCl (Merck, Darmstadt, Germany) prepared as 50 mM/L for dissolution of metabolites. Concentrations of melamine (0.62, 1.25, 2.5, 5 and 10mM), cyanuric acid (0.94, 1.86, 3.75,7.5 and 15mM) and uric acid (0.062, 0.125, 0.25, 0.5 and 1mM) were prepared with Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.4).

UV-Absorbance Spectrophotometry

Absorbance spectra were carried out using a UV-absorbance spectrophotometer (Epoch-2, BioTek, Vermont, USA). After the absorption spectra of melamine and its metabolites, their mixtures with ct-DNA and *S. aureus* DNA were determined. The mixtures with fixed concentration of DNA and varyin concentrations of melamine, cyanuric acid or uric acid were incubated at 37 °C for 60 minutes. Finally, at the end of incubation, the wavelength range (λ range) was set at 240–320 nm and the absorbance spectrum measurements were performed.

Fluorescence Spectrophotometry

Mithras[2] LB943 multimode fluorescence spectrophotometer (Berthold Technologies, Bad Wildbad, Germany) and 96 well plates (237105, Thermo, Waltham, USA) were used to monitor fluorescence intensity changes. DNA+melamine/metabolite combinations were prepared for both types of DNA and incubated at 37 °C for 60 minutes. After the incubation, the samples were stained with 10 µl of SafeView™ Classic nucleic acid dye (G108, Applied Biological Materials Inc. (abm), Richmond, Canada) and fluorescence readings were

performed. The excitation value (λex) was 485 nm and the wavelength range (λrange) was 500-600 nm for the measurement.

Agarose Gel Electrophoresis

Agarose gels (1.5%, w/v) were prepared with 1x TBE buffer solution (10.8 g tris, 5.5 g boric acid and 0.93 g EDTA per liter, pH 8.0). After preparation of different concentrations of melamine and its metabolites, their combinations with eukaryotic and prokaryotic DNAs were incubated at 37°C for 60 minutes. DNA+ melamine/metabolite combinations (14 µL) were mixed with 2 µl DNA loading dye and 2 µl EZ Vision™ DNA dye (Amresco, N472-KIT, Ohio, USA) and loaded into gel wells. Electrophoresis was performed in the presence of TBE buffer solution at 100 V for 1.5 hours. After electrophoresis, the gels were visualized by Gel Doc EZ Gel Documentation system (Bio-Rad, Hercules, USA) and the intensity of the DNA bands were analysed using Image Lab 6.0 software (Bio-Rad, Hercules, USA).

CONCLUSIONS

With the present *in vitro* study, it was determined that melamine and its metabolites which can be added to both human and animal foods to reveal fake high protein content, exhibited groove binding with the prokaryotic and eukaryotic DNA molecules. Although it has been reported that this binding may cause structural damage in DNA, studies on the possible mutagenic effect are limited, and the lack of data on mutagenicity in humans indicates that it is an area open to research.

With this study, it was concluded that the data obtained on the *in vitro* interaction of genomic DNA+melamine/metabolites will form the basis for future studies to be designed with *in vivo* experiments to understand the genotoxic effects of the tested molecules.

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