GENOTOXIC AND CYTOTOXIC ACTIVITY OF CYANOBACTERIAL (BLUE-GREEN ALGAL) TOXIN BMAA IN HUMAN LYMPHOCYTE CELLS

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Abstract

Cyanobacteria, one of the oldest life forms, are also known as blue-green algae. Many species of cyanobacteria produce metabolites called cyanotoxin, which are classified as hepatotoxins, neurotoxins and cytotoxins and are highly toxic to vertebrate organisms. One of these toxins is the Beta-N-methylamino-L-alanine (BMAA). Blooms (large numbers or colonies) of the cyanobacteria or related organisms produce one or more toxins that can be dangerous to fish, wild animals and humans. There is not enough information in the literature related to the genotoxic effect of this toxin. In this study, it was aimed to determine investigation of the cytotoxic and genotoxic activity of cyanobacterial toxin BMAA in human lymphocyte cells by micronucleus assay. Different concentrations (1, 5, 10 and 20 μg/mL) of BMAA cyanotoxin were applied to human lymphocyte cell culture and micronucleus frequencies (MN) and nuclear division index (NDI) were calculated to determine genotoxic and cytotoxic effects. The data obtained from our study were compared with the negative control group prepared with dimethyl sulfoxide (DMSO) and with the well known ethyl methanesulfonate (EMS) with genotoxic effect and positive control group. According to these results; increasing the frequency of micronucleus according to the whole control negative control group that we used BMAA was not statistically significant compared with the EMS (positive control group) (p>0.05). However, no significant cytotoxic effect was found when the results of the NDI were examined (p>0.05).

Keywords: Cyanobacteria, Genotoxicity, BMAA, Micronucleus.

1. Introduction

In our study, we aimed to investigate the genotoxic and cytotoxic effects of Beta-N-Methylamino-L-Alanine (BMAA) cyanobacteria toxin on human lymphocyte cells using micronucleus test technique. At least 46 species of cyanobacteria, called blue-green algae due to the photosynthetic pigments they contain, and present in the terrestrial and coastal waters, show toxic effects on vertebrates. These toxins are classified as hepatotoxin, neurotoxin and cytotoxin in terms of their effects and they are chemically analyzed in three groups as cyclic peptides (hepatotoxic, microcystin and...
nodular), alkaloids (neurotoxic, anatoxin and suctytoxin) and LPS (lipopolysaccharide) endotoxins. One of these toxins is BMAA (Novak et al., 2016).

BMAA is a small amino acid (NPAA) class of more than 900 non-protein amino acids (NPAA) produced by plants, fungi and microorganisms, with the chemical formula C₄H₁₀N₂O₂ and a molecular weight of 118.13 g/mol (Jonasson et al., 2008). BMAA is a non-protein peripheral amino acid that has been suggested to be associated with neurodegenerative disease (Cox et al., 2017). Drinking water containing cyanobacterial toxins, aspiration and contact with these waters, is the cause of their effects. It has been reported that, in poisonings, especially children and those with liver disease and renal insufficiency carry more risk due to their more water consumption than body weight (Geh et al., 2016). Chronic effects of cyanobacteria hepatotoxins were examined to stimulate tumor development and lead to the formation of liver cancer (Fleming et al., 2002). In the aquatic environment, organisms can be exposed to a variety of substances resulting in chromosomal aberrations that can result in cancer and cell death (Svirčev et al., 2014; Cavalcante et al., 2003). It is important to find out the dangerous effects of these substances on the aquatic and terrestrial environment and on humans.

Genetic toxicology, a subdivision of toxicology, is a science that delves into changes in the DNA molecules of a cell during normal biological processing of the organism or depending on chemical, physical, and biological factors (Young, 2002; Gibbons and LeBaron, 2017). "Genetic toxicity" or "genotoxicity" is a generic term that encompasses damages such as DNA attachments, DNA breaks, gene mutations, chromosomal structural abnormalities, and aneuploidy that occur in the nucleus, chromosome and DNA structure. Interacting with enzymes that enable the copying of the DNA or genome, and causing mutation, the genotoxic substances damage to DNA or cause some alteration, which is called as "genotoxic effect" (Zeiger, 2004).

The micronucleus test, one of the genotoxicity tests, is a short-term test technique widely used to determine the clastogenic and aneugenic activities of various chemical substances and physical agents in mammals (Sekeroglu and Sekeroglu, 2011). Lymphocytes are considered suitable cells to investigate mechanisms of genotoxic action such as DNA damage, chromosomal abnormalities and MN formation. The 'Cytokinesis block' micronucleus (MN) method applied in human peripheral blood lymphocytes is a standard cytogenetic test among the genetic toxicology tests accepted by many laboratories around the world (Güven et al., 2006). In the micronucleus test, which is one of the short-term genotoxicity tests, it was determined that the micronucleus formed as a result of chemical application consisted of a complete chromosome which could not be separated from the ascentic chromosome fragments and/or spindle threads that appeared during the mitosis of the cell and could not migrate to the poles (Fenech, 2000). According to Fenech (2000), the nuclear membrane is formed around chromosomes and/or fragments that are separated in the telophase and thus micronuclei are formed smaller than the parent nucleus. Appearance and each individual affiliation must be given in a separate row together with the e-mails of the authors. If there are more than one author who have the same affiliation, their e-mail addresses should be given in order of their names. There must be an 8 pt space between the last e-mail address and the reception/acceptance date information of the paper.

2. Materials and Methods

2.1. Material

Peripheral blood was used as material throughout the study, from healthy men and women, aged 23-25 years, who did not use cigarettes and alcohol, did not have infectious disease recently, weren’t exposed to any physical agent such as X-rays.
2.2. Preparing the cell culture and preparates

6 mL of medium (Chromosome Medium B) prepared beforehand and brought to 37 ºC was placed in sterile cell culture tubes. 12 drops (0.5mL) of blood was dropped on each tube. From the cyanobacterial toxin solutions prepared at the specified concentrations, 0.25mL was added to the cultures in the tubes and mixed well. The tubes were allowed to incubate for 72 hours at 37 ºC in the oven. Forty-eight hours after the start of the incubation, Cytochalasin-B was added to all culture tubes as a final concentration of 3μg/mL. The tubes were again put in the oven (at 37ºC) to complete the 72 hour incubation period. Tubes removed from the oven at the end of 72 hours were centrifuged at 1000 rpm for 10 min. After centrifugation the supernatant was discarded with a pastry pipette. 5-6 mL of hypotonic solution was slowly added onto the remaining pellets of the tubes. Tubes with hypotonic solution were incubated for 25 min at 37 º C. At the end of the period, the tubes were centrifuged again at 1000 rpm for 10 min and the supernatant was discarded in the same manner. After supernatant was removed, 7mL of freshly prepared cold detection solution was vortexed onto the pellets remaining in the tubes, and centrifuged at 1000 rpm for 10 min and the resulting supernatant was removed by drawing with a pastry pipette. The last step is repeated 2-3 times on average until the image in the tubes is clear. The supernatant in the tubes was finally discarded and the remaining pellet was mixed by pipetting with a pastry pipette. The pellet taken with pipette was dropped from about 20-25 cm height on the slides written protocol number and held in the cold (+4°C) detection solution. Preparations prepared in this way were left to dry for 3 days. At the end of the third day, the well-dried preparations were kept in the Giemsa stain for 15 min. From the preparates prepared from each individual, 1000 pieces of two-nucleus (binuclear) cells were counted, from which micronuclei were identified and recorded.

In addition, to determine the possible cytotoxic effects of cyanobacterial toxins, 1000 cells were counted from preparates prepared from the same person, and the ratio of one, two, three and four nuclei among these cells was determined. Moving from this proportion, the Nuclear Division Index (NDI) was calculated (Fenech, 2000). For the statistical analysis of micronucleus (MN) and NDI values obtained from our studies, SPSS (Statistical Package for the Social Sciences) 13.0 program was used. Univariate analysis of variance (ANOVA) and Tukey tests were applied to compare the data obtained between distilled water control and treatment groups.

Figure 1. a) Cells not bearing micronucleus  b) Binucleated cells bearing single micronucleus (400x)
Figure 2. a) Binucleated cells bearing dual micronucleus (MN) and b) the ones bearing three micronuclei (MN) (400x)

3. Results and Discussion

Beta-N-methyl-amino-L-alanine (BMAA) algal toxin and EMS were administered to peripheral blood cultures prepared with blood from four different donors at different doses (1, 5, 10 and 20 μg/ml) and the resulting micronucleus (MN) frequencies and Nuclear Division Index (NDI) data are presented in Table 1. In addition, the statistical data that resulted from comparing all the application groups with the DMSO (negative control group) are given in the same charts. While MN frequencies were being obtained, a total of 4000 binucleated cells from four donors, 1000 from each donor, and 1000 cells randomly selected from each donor for NBI were examined.

Table 1. The micronucleus and nuclear division index values obtained after Beta-N-methyl-amino-L-alanine application

<table>
<thead>
<tr>
<th>Application Groups</th>
<th>Concentration (µg/mL)</th>
<th>Numbers of Binucleated Cells</th>
<th>MN Frequencies in Binucleated Cells</th>
<th>Average Number of MN ±S.E.</th>
<th>Nuclear Division Index (NDI) ±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1) (2) (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>4000</td>
<td>9</td>
<td>1</td>
<td>0.275±0.06</td>
</tr>
<tr>
<td>EMS</td>
<td>0.7</td>
<td>4000</td>
<td>71</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Beta-N-methyl-amino-L-alanine</td>
<td>1</td>
<td>4000</td>
<td>9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4000</td>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4000</td>
<td>18</td>
<td>2</td>
<td>1</td>
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<td></td>
<td>20</td>
<td>4000</td>
<td>22</td>
<td>2</td>
<td>0</td>
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MN: Micronucleus; EMS: Ethyl methanesulfonate; S.E.: Standart Error; *: The difference between the groups is significant at p <0.01.

In addition, photographs of binucleated cells not bearing micronucleus and of the ones bearing a single micronucleus are shown in Figure 1, and the photographs of binucleated cells bearing 2 or more micronuclei are shown in Figure 2.

While the mean amounts of MN obtained by Beta-N-methyl-amino-L-alanine (BMAA) at different doses (1, 5, 10 and 20μg/ml) were determined that 0.225±0.04; 0.325 ± 0.03; 0.775±0.06 and 0.825±0.03, respectively, these rates were found to be 2.575±0.31 in the (EMS) positive control group and 0.275 ± 0.06 in the (DMSO) negative control group (Table 1). The increase in MN frequencies detected in binucleate cells also indicates the genotoxic effect of the applied substance. When examining the MN frequencies in the binucleated cells examined after all treatments, triple MNs were only
observed in the EMS positive control group and in the 10 μg/mL BMAA administration group. In all other application groups, it was determined that there were single MNs (Table 1).

When Table 1 was examined, although the mean number of MN was increased in all treatment groups except the 1 μg/mL administration group, these results were statistically insignificant at p>0.01 level in all groups when compared with the negative control group. Nuclear division index (NBI) values were also calculated in our study to determine possible cytotoxic effects of algae toxin. In Table 1, when the effect of BMAA on NBI was examined, it was observed that there was no decrease in this value in any of the treatment groups except for the last two treatment groups and accordingly there was no cytotoxic effect.

Beta-N-methylamino-L-alanine is an amino acid group produced by many species of blue-green algae. It has been discussed for many years that BMAA exposure may be a cause of neurological diseases such as ALS, Parkinson's and Alzheimer’s. Numerous in vitro and in vivo experiments have shown that BMAA can cause motor dysfunction by damaging motor neurons. For example, in vivo studies with rats have shown that motor function abnormalities such as stiffness, ataxia weakness and contraction occur after BMAA exposure (Chang et al., 1993; Matsuoka et al., 1993; Dawson et al., 1998). However, such effects are likely to be caused by damage such as vacuolization of motor neurons (de Munck et al., 2013), mitochondrial dysfunction, and degeneration of hippocampal neurons (Karlsson et al., 2009b, 2011 and 2012). However, many studies emphasize that the mechanism of neurotoxicity caused by BMAA is still not fully understood.

Similar studies with mice showed that BMAA had similar motor effects to that of rats (Smith and Meldrum, 1990) and were observed symptoms such as, body movement, uncoordinated limb, urinary and fecal incontinence, and dyspnea (Al-Sammak, 2012). Other neurotoxic effects of BMAA on mice have been reported to include acute locomotive activity disorders, hyperactivity (Karlsson et al., 2009a), neurodegeneration of hippocampal neurons (Buenz and Howe, 2007) and retinal neuron death (Santucci et al., 2009).

In another study, toxic effects of BMAA on fruit fly (Drosophila melanogaster) were investigated (Zhou et al. 2009). As a result of the study, it has been observed that life-shortening toxic effects occur in individuals. As a result of other studies of the same researchers, it has been found that BMAA can accumulate in brain tissue in D. melanogaster and cause problems such as decreased fertility in females and impaired learning and memory ability (Zhao et al., 2010). Okle et al. (2013) analyzed the toxic effects of Beta-N-methyl-amino-L-alanine on Apis mellifera. Researchers have finally found that BMAA has effects that increase the formation of reactive oxygen species in bee brains and reduce in ability of individuals to learn smell. The authors emphasized that observations of motor dysfunction in fruit flies and odor learning impairment in honey bees are important evidence of BMAA-induced neurotoxicity.

Esterhuizen et al. (2011) studied the effects of Ceratophyllum demersum, an aquatic macrophyte species, against oxidative stress responses. Consequently, they found that BMAA had a important inhibition effect on oxidative stress enzymes tested, including catalase, glutathione reductase, glutathione peroxidase, guaicol peroxidase, and superoxide dismutase (Esterhuizen-Londt et al., 2011b). The authors suggest that the mechanism of toxicological action of Beta-N-methyl-amino-L-alanine is to induce oxidation by inhibiting oxidative stress enzymes (Esterhuizen-Londt et al., 2011b). In another study that investigated toxicity of Beta-N-methyl-amino-L-alanine in Daphnia magna; although there is no lethal effect on Daphnia, it has been found to decrease mobility and negatively affect the longevity characteristics such as reproduction and proliferation (Lürling et al., 2011).
As a result of a study investigating the effects of BMAA on aquatic vertebrates; BMAA has been shown to exhibit toxic effects, such as heart rate, pericardial edema, abnormal spinal axis formation, clonus-like convulsions, and developmental abnormalities, including zebra fish (Danio rerio) during early life development (Purdie et al., 2009).

In short, many studies have demonstrated that BMAA is neurotoxic for many organisms in both terrestrial and aquatic environments. However, there are not many studies on genotoxic effects in the literature. A study investigating the genotoxic effect of different Salmonella typhimurium strains using Ames test, similar to our results, found a study that was not cytotoxic or genotoxic for bacteria (Novak et al., 2016).

In this respect, the results of our study in which the genotoxic effect of BMAA has been investigated in human organism are very important.

4. Conclusion

The genotoxic and cytotoxic activity of β-N-Methyl-Amino-L-Alanine (BMAA) has been investigated for the first time in our study. As a result of our study, we concluded that BMAA does not have genotoxic and cytotoxic effects in human lymphocyte cells but should be supplemented by in vivo and in vitro studies in other eukaryotic organisms.

5. Acknowledgements

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6. References


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