THE ALKALINE COMET ASSAY USED IN EVALUATION OF GENOTOXIC DAMAGE OF DRINKING WATER DISINFECTION BY-PRODUCTS (BROMOFORM AND CHLOROFORM)

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The single cell gel electrophoresis (SCGE)/comet assay combines the simplicity of biochemical techniques for detecting DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites and cross-linking with the single cell approach typical of cytogenetic assays. The advantages of the SCGE technique include:

• the collection of data at the level of the individual cell, allowing for more robust types of statistical analyses;
• The need for small numbers of cells per sample (<10,000);
• its sensitivity for detecting DNA damage;
• that virtually any eukaryotic cell population is amenable to analysis.
The alkaline comet assay has been used as a useful method for monitoring genotoxic effects of environmental pollutants in the root nuclei of *Allium cepa* and various plants which allows the detection of single- and double-strand breaks, incomplete excision-repair sites and cross-links. It has been introduced to detect even the small changes in DNA structure. It is a technically simple, highly sensitive, fast and economic test. The alkaline comet assay on plants especially on *Allium cepa* have slightly differences from the assay with the other types of eukaryotic cells (*Saccharomyces cerevisiae* or lymphocytes) in the composition of different solutions used which will be exposed in materials and Methods section.

*Allium cepa*
Trihalomethanes (THMs) are well known disinfection by-products of water by chlorine and other oxidants. **Bromoform and chloroform are the most prevalent trihalomethanes in drinking water in all over the world.** Many studies reported a relationship between the presence of these compounds and many different types of cancer cases in the studied sectors as well as birth-outcomes cases, fetus anomalies and many other genital anomalies.
• The aim of this study was to evaluate the DNA damage caused by these two compounds.

• Alkaline comet assay on *Allium cepa* roots cells was employed in testing the hypothesis.
MATERIALS AND METHODS

In the protocol used to detect DNA damage:

**Organism:**

*Allium cepa* (2n=16), onion, bulbs, 25–30 mm diameter, without any treatment, were purchased from a local supermarket in Turkey.

**Chemicals:**

Bromoform \((\text{CHBr}_3)\) 99 % (CAS No. 53-23-3), Chloroform\(\text{CHCl}_3\) 99.2 % (CAS No. 67-66-3), methyl methanesulfonate (MMS, CAS No.67-27-3), normal melting point agarose (NMPA), low melting point agarose (LMPA), di-sodium salt of ethylene diamine tetra acetic acid (EDTA), Tris buffer, ethidium bromide (EtBr), Trizma base, Tris HCl, Triton X-100 and SDS were purchased from Sigma Aldrich (Munich, Germany).
Exposition of *Allium cepa* onion bulbs to different concentrations of bromoform and chloroform for 24 h
Nuclei isolation by cutting roots on ice 500 µl Tris- MgCl₂ ice-cold buffer (0.2 M Tris, pH 7.5; 4 mM MgCl₂-6H₂O; 0.5% w/v Triton X-100)
Step 3

Filtration realised on Nytre1® polyamide of 20µm, to eliminate plant debris.
Step 4

Preparing the Microscopic slides

1% NMP, drying.

20 µl of Nuclei suspension +100µL of 0.8% LMP, drying.
Lysis for 1h at 4°C in ice-cold lysing solution (1 M NaCl; 30 mM NaOH, 0.5% w/v SDS, pH 12.3)
Step 6

Relaxation for 1 h on electrophoresis buffer (30 mM NaOH and 1.5 mM EDTA, pH 12.3)
Running the material on Electrophoresis for 20 min at 25 V (1Vcm⁻¹) and 300 mA at 4°C
Neutralisation and Dyeing: 50 μL of ethidium bromide (BET; 20 μg/ml) for 5 min
Step 9

Observing them on Fluorescence Microscope
Step 10

Statistical analysis of the data

For this, Duncan multiple range tests were performed by using one-way analysis of variance (ANOVA) on SPSS 15.0 version for Windows software.
RESULTS

Results are summarized in Table 1. Comet assay results showed that DNA damages were significantly higher at different concentrations of bromoform and chloroform compared to the negative control. The values for bromoform concentrations of 75 µg/mL and for chloroform concentrations of 100 µg/mL are higher than the positive control.

Table 1: Detection of DNA damage in nuclei of *A. cepa* root meristems exposure to Bromoform and Chloroform using the Comet assay

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Doses (µg/ml)</th>
<th>DNA Damage (Arbitrary Unit ±SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>-</td>
<td>3.5±0.7a</td>
</tr>
<tr>
<td>MMS</td>
<td>10</td>
<td>13.5±2.12b</td>
</tr>
<tr>
<td>Bromoform</td>
<td>25</td>
<td>7.5±0.7c</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10.5±0.7d</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>15.5±0.7b</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>21±1.41e</td>
</tr>
<tr>
<td>Negative control</td>
<td>-</td>
<td>3.5±0.7a</td>
</tr>
<tr>
<td>MMS</td>
<td>10</td>
<td>13.5±2.12bc</td>
</tr>
<tr>
<td>Chloroform</td>
<td>25</td>
<td>13±2.82bc</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>11.5±2.21b</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>17±2.82bc</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>15±0.01c</td>
</tr>
</tbody>
</table>

* Means with the same letter do not differ statistically at the level of 0.05.

SD: Standard Deviation
This could be due to DNA damage induced by oxidative stress. The secondary genotoxicity causing potential of the agents could be due to the production of reactive oxygen species or lipid peroxidation, or both. It is notable that, for the test compounds employed in this study, secondary genotoxicity is associated with high concentrations approaching the level for cytotoxicity and may explain why they are only weakly positive in certain genotoxicity assays at high concentrations (Luo et al. 2004). A couple of studies showed a dose-dependent increase in DNA double strand breaks by chloroform that occurred prior to cytolethality (Davidson et al., 1982; Beddows et al., 2003).
It also increased the formation of DNA strand breaks and lipid peroxidation at non-cytotoxic concentrations. This increase mirrored the elevation in lipid carbonyls and suggests that chloroform can induce indirect DNA damage as a consequence of lipid peroxidation (Beddows et al. 2003). Other studies have shown that THMs, including chloroform, also yield CO as a metabolite. The order of yield of CO was greatest for bromoform chloroform for the same dose (Davidson et al. 1982). There is indication that brominated DBPs may be more carcinogenic than chlorinated analogs (WHO 2000).
The Allium test was simultaneously adapted for detecting low levels of DNA damage through comet assay (Chakraborty et al. 2009; Liman 2011; 2013). The measurement of DNA damage in the nuclei of higher plant tissues is a new area of study with SCGE. This assay could be incorporated into *in situ* monitoring atmosphere, water and soil: the comet assay allows a fast detection without any need to wait for progressing mitosis (Cotelle and Ferard 1999; Poli et al. 1999).
CONCLUSION

• The measurement of DNA damage in the nuclei of higher plant tissues is a new area of study with SCGE.
• This assay could be incorporated into *in situ* monitoring atmosphere, water and soil: the comet assay allows a fast detection without any need to wait for progressing mitosis.
• Based on the results we can conclude that the classical *Allium* test can give a more comprehensive data when done in combination with comet assay, which is faster and simpler.
Genotoxicity of drinking water disinfection by-products (bromoform and chloroform) by using both *Allium* anaphase-telophase and comet tests

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THANK YOU FOR YOUR ATTENTION...