COMPARISON BETWEEN ALKALINE AND NEUTRAL VARIANTS OF YEAST COMET ASSAY

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ABSTRACT
Comet assay gain its popularity because it is a fast and relatively easy-to-perform method for assessing damages in DNA. The two persisting variants of the method: alkaline and neutral, lead to some doubts which one would be suitable for a certain purpose. Here we present a comparison between the two versions of the developed by us Yeast Comet Assay (YCA). Since DNA damages can be quite diverse, i.e. single- or double-stranded cuts, oxidative damages, photodimers, etc., we warn that the method of YCA has to be applied with attention and a certain level of understanding concerning the exact damage that is attempted to be assessed.

Keywords: yeast, Comet assay, genotoxins

Introduction
The Comet assay is an elaborative technique for detection of damages in the molecule of DNA at a single-cell level. The method can differentiate among plenty of damages, including single- and/or double-strand DNA breaks, photodimers, alkali labile sites, covalent shifts between bases, etc (1). Two variants of the Comet assay method exist: the initially developed neutral Comet assay (6) and the later modified variant in high pH (8). It is commonly suggested that both variants hold the potential to distinguish between different DNA breaks. In the neutral variant the molecule of DNA is preserved as a double-stranded structure which leads to uncovering of double-stranded DNA breaks (9). In the alkaline variant of the method the denaturing step allows to reveal simultaneously double- and single-stranded DNA breaks, as well as alkali labile sites, which leads to the general assumption that the alkaline assay is much more sensitive in comparison with its neutral variant in DNA damage detection (5). However, since one break per loop in the DNA molecule leads to chromatin loop relaxation, the neutral version can be considered as quite sensitive when lower concentrations of single-strand DNA cutting agents are applied (7).

We have applied the method of Comet assay on the single-cell eukaryotic organism - *Saccharomyces cerevisiae*. Interestingly, the yeast cells showed higher sensitivity to the action of the studied genotoxins (4). The new method was called Yeast Comet Assay (YCA), apparently exhibiting higher sensitivity in studying of DNA damage (5). Recently, we extended our research and developed the neutral version of the YCA (2). In the present study we show data of comparison between alkaline and neutral version of the YCA. The results show that generally the neutral version of YCA is more sensitive than the alkaline for the compounds tested. Our results allow us to underline the fact that both methods should be used in order to reveal lower concentrations of genotoxins and a lot of precautions should be taken for obtaining reproducible results.

Materials and Methods
All chemicals in this study were purchased from Sigma-Aldrich unless stated otherwise.

Yeast strain
The yeast *Saccharomyces cerevisiae* strain 3A: MATa/α, *gal*1, *leu*2, *ura*3-5 was used in this research.

Yeast Comet Assay (YCA)
Yeast cultures were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C to middle logarithmic phase (*OD*600 = 0.5 – 0.6) and treated in the medium *in vivo* for 2 hours with different concentrations of the compounds. The concentrations encompass a broad range starting from 0,001 mM to 1mM.

Alkaline YCA
The alkaline Yeast Comet Assay was applied as was developed and published elsewhere (4) with minor modifications. Yeast cells were collected by centrifugation, washed and resuspended in S-buffer (1M Sorbitol, 25mM NaH2PO4, pH 6.5). Aliquots from the cell suspension were mixed with low-melting agarose to a final concentration of 0.7%, containing Zymolyase and were spread as micro-gels on microscopic slides. The microgels were incubated for 5 minutes at 4°C in order to set the gells and after that for 10 minutes at 37°C for spheroplasting of yeast cells. After this incubation the slides were submerged in lysis solution (1M NaCl, 50mM EDTA, 30mM NaOH, 0.1% N – lauroylsarcosine; pH 10) for 1 hour at 4°C and afterwards were washed 3 times for 20 minutes at 4°C with denaturing solution (30mM NaOH, 10mM EDTA; pH 12.6) and subsequently subjected to electrophoresis (0.45 V/cm) in the same denaturing solution for 15 minutes.
Neutral YCA

All steps before the lysis procedure were the same as those in the alkaline YCA. Next, the slides were placed in a cold lysis solution (146mM NaCl, 30mM EDTA, pH 8, 1mM Tris-HCl, pH 8, 0.1% N-lauroylsarcosine; pH 9) for 20 min and then washed in TBE buffer, pH 8.3 for 3x5 min. Electrophoresis was conducted at 4°C for 15 minutes at 0.45 V/cm in electrophoresis buffer (TBE; pH 8.3) (2).

After electrophoresis, the gels were dehydrated in 75% and 95% ethanol for 5 minutes each. Finally, the slides were left to air dry and observed under fluorescent microscope by staining of the DNA with SYBR green (Molecular probes).

Compounds

For treatment of yeast cells we used the following compounds (Table):

<table>
<thead>
<tr>
<th>Tested compounds</th>
<th>CAS number</th>
<th>E number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium nitrite</td>
<td>7632–00–0</td>
<td>E 250</td>
</tr>
<tr>
<td>Caffeine</td>
<td>58–08–2</td>
<td>no number</td>
</tr>
<tr>
<td>4-aminoantipyrine</td>
<td>83–07–8</td>
<td>no number</td>
</tr>
<tr>
<td>Indigocarmine</td>
<td>860–22–0</td>
<td>E 132</td>
</tr>
<tr>
<td>Erythrosine</td>
<td>16423–68–0</td>
<td>E 127</td>
</tr>
<tr>
<td>Fast Green</td>
<td>2353–45–9</td>
<td>E 143</td>
</tr>
</tbody>
</table>

Results and Discussion

Characterization of alkaline and neutral yeast comets

By using *Saccharomyces cerevisiae* as a model organism we developed the method called Yeast Comet Assay (YCA), which turned out to be more sensitive to the action of genotoxins (4). Later on, we developed the neutral variant of the method. In order to characterize these two methods better we treated yeast cell cultures with six different substances. These substances were chosen because of their application in food and/or pharmaceutical industries as food additives or drug compounds (see Table 1). *S. cerevisiae* cells from early logarithmic phase of growth were treated *in vivo* with the above-mentioned compounds (Table 1) for 2 hours at 30°C and then were subjected to alkaline and neutral YCA. All of the above substances gave comets after treatment of the cells for two hours. Two representative comets from yeast cells obtained by the alkaline and neutral YCA are shown on Fig. 1. Some important differences can be easily observed by a well-trained eye. In good concordance with our previous experience with the alkaline version of YCA, in this study the yeast comets appeared more heterogeneous under alkaline conditions of the method rather than homogenous when compared with comets of higher-eukaryotic cells. Many blobs and granules can be seen in the tails (Fig.1 A). These granules most probably represent fragments of yeast chromatin, realized under the electrophoresis step, and reflect the weak structure of yeast nucleus. On the other hand, yeast comets, obtained in neutral conditions (2) and compared with the alkaline yeast comets, look more uniform with strings of chromatin loops easily seen in their tails (Fig.1 B). According to us and other authors (3), the comets’ appearances obviously reproduce differences in the compaction of the DNA molecule in the yeast nucleus, differently revealed under the two conditions of the YCA.

Comparison between alkaline and neutral YCA sensitivity

Generally in the field of Comet assay, the question about the sensitivity of both variants, alkaline and neutral Comet assay methods (1), is widely discussed. For practical reasons it is of great interest to compare the sensitivity of both variants. Moreover, for the method of Yeast Comet Assay it has not been done yet. Therefore, yeast cells were treated with the same concentration gradient of the tested substances for both variants of the YCA. After that, the minimal concentration of the genotoxin leading to DNA damage, i.e. 70% of comets in the gels, was assessed and analyzed under both variants of the YCA, thus giving information about their sensitivity. This comparison is present on the graphic in Fig. 2. The data represents the results from three independent repetitions of the experiments and hence reflects statistically significant observations.
At first sight, it is obvious that the neutral variant is more sensitive than the alkaline, leading to almost 70% of comets in the gels after treatment with lower concentrations of the studied substances in comparison with the alkaline YCA, where the same percentage of comets in the gels was obtained after treatment with higher concentrations of the same substances. However, closer examination shows that the better sensitivity of the neutral variant depends on the used genotoxin. Indeed, for four of the examined genotoxins (4-AAP, indogocarmine, erythrosine and fast green) the neutral variant was 10 times more sensitive in comparison with the alkaline YCA, however for the other two (caffeine and sodium nitrite) there was no difference in the concentrations of the these substances, that lead to 70% of comets in the two variants.

Having in mind that some of the genotoxins do not induce cuts but other changes in the molecule of DNA (see above), we warn that the method of YCA has to be applied with great attention and with good understanding of what exactly damage is attempted to be assessed. There is an immense need for more and wider experiments for an unambiguous determination of the neutral comet assay as a better method of choice for the genetic toxicology.

Acknowledgments
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REFERENCES