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ASSESSEMENT OF ACROLEIN-INDUCED CELLULAR DAMAGE IN THE YEAST *SACCHAROMYCES CEREVISIAE* CELLS USING MICROSCOPY TECHNIQUES

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Abstract

Acrolein is well-known and commonly found a compound showing cytotoxic activity. This study was aimed at a comparison between numerous tests based on the technique of bright-field and fluorescence. We applied the non-fluorescent dye PhloxineB or the single or double staining procedure with specific fluorescent dyes like fluorescein diacetate (FDA), propidium iodide (PI), FUN-1, 4',6-diamidyno-2-fenyloindol (DAPI), MitoTrackerGreen, Rhodamine-Phalloidin and immunofluorescence technique to assess the viability, metabolic activity, structural changes and oxidative protein damages in the yeast *Saccharomyces cerevisiae* cells exposed to acrolein. The comparison of the results showed that the microscopic methods are a simple and very useful research tool for testing the toxicity of chemicals at the cellular level.

Keywords: *yeast, acrolein, oxidative stress, fluorescence, microscopic methods*

Introduction

Fluorescence microscopy is an essential and very important technique used to assess viability and vitality of the cells, visualization of cellular organelles and localization of macromolecules damages. There are many fluorescent dyes specific for various intracellular structures e.g. DAPI to visualize nuclei, MitoTrackerGreen or RhodamineB to label mitochondria, Rhodamine-Phalloidin to visualize actin cytoskeleton, MDY-64 to label membrane of vacuole or to identify the metabolic activity of the cell e.g. FUN-1 or fluorescein diacetate (FDA). Moreover, the use of fluorescently labeled antibodies allows for the localization of the selected macromolecules. These methods can be especially usefull in toxicological studies.

Cytotoxic substances, which include a number of chemical compounds common occurring in the environment or food products show a multidirectional action in the cell. Disorders which result from their actions affect both the structural elements of the cell and the activities of numerous metabolic pathways. The cell response depends on the concentration and time of exposure of the toxic compounds and it may be: the cell cycle arrest, changes of the metabolic activity and/or intracellular structures of the cell and the cell death induction by necrosis or apoptosis. An example of this type of compound is acrolein, chemically classified to the unsaturated aldehydes. It is a common ingredient / cause of environmental pollution as a by-product the incomplete combustion of petrol or the basic fuel materials as carbon or wood. It is also compound of the tobacco smoke (**Caffaro-Filho *et al.*, 2010**). This aldehyde is also found in many types of food undergoing heat treatment such as animal and vegetable fats (**Abraham *et al.*, 2011**), but also in natural products as vegetables and fruits. It was reported that the toxicity of acrolein involves enhancement of cellular oxidative stress by depletion of glutathione content/level, generation of reactive oxygen species, macromolecules damages (mainly protein and DNA modification) and structural alteration on actin cytoskeleton and mitochondrial network (**Kwolek-Mirek *et al.*, 2009, Kwolek-Mirek and Bartosz, 2011**).

In this paper we present various methods for studying the toxicity of acrolein based on the fluorescence microscopy, using a simple eukaryotic model of the yeast *Saccharomyces cerevisiae*.

Material and methods

Yeast strains

The following yeast strains were used: wild-type SP4 MATα *leu1 arg4* (**Bilinski *et al.*, 1978**), and Δ*sod1* mutant, isogenic to SP4, MATα *leu1 arg4 sod1::natMX* (**Koziol *et al.*, 2005**).

Media, growth conditions and condition of incubation

Yeast was grown in a standard liquid medium (YPD; 1% yeast extract, 1% yeast bacto-peptone, 2% glucose) on the shaker (150 rpm/min), at a temperature 28°C.

Cells from exponential phase culture were centrifuged, washed twice, suspended to a final density of 10^8 cells/ml in 100 mM phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.1% glucose, and incubated with shaking for 60 min with 0.4 mM allyl alcohol (*precursor of acrolein in the cell)* (**Bilinski *et al.*, 2005**), at 28°C.

Viability assays

For verification of budding cells, after incubation with allyl alcohol 5 µl of cell suspension were spotted on the plate with solid YPD medium. At the start of the experiment and after 24 h of culture. Cultures were grown at 28°C.

Viability of cells was estimated with 10 µg/ml PhloxineB (2 mg/ml stock in MilliQ water) (**Minois *et al.*, 2005**). After incubation the cells were collected by centrifugation, washed twice and resuspended in phosphate-buffered saline (PBS). For image acquisition was used OLYMPUS BX-51 fluorescence microscope equipped with a DP-72 digital camera and analysis software Cell^D.

Fluorescent dyes and antibodies

Fluorescein diacetate (FDA) and propidium iodide (PI) were from Sigma-Aldrich (Poznan, Poland), 4',6-diamidyno-2-fenyloindol (DAPI), FUN-1, MitoTrackerGreen, Rhodamine-Phalloidin were from Molecular Probes (Eugene, OR, USA), primary polyclonal rabbit antibody specific to the 2,4-dinitrophenol-hydrazine (DNP) moiety of the proteins (ab6306) and goat anti-rabbit conjugated with Chromeo™ 546 secondary antibody (ab60317) were from Abcam (Cambridge, UK).

Fluorescence microscopy

After incubation the cells were collected by centrifugation, washed twice and resuspended in appropriate medium depending on the staining procedure. Cell suspension contained about 1×10^7 cell/ml were stained in appropriate conditions shown in Table 1 and according to the manufacturer's protocols (Molecular Probes). For image acquisition was used OLYMPUS BX-51 fluorescence microscope equipped with a DP-72 digital camera and analysis software Cell^D.

Table 1

Yeast cell staining conditions

Fluorochrom	Dye concentration in sample	Medium	Incubation time [min]	Staining temperature [°C]	λ_{ex} [nm]	λ_{em} [nm]
FDA/PI	10 μg/ml/5 μg/ml	PBS	15	RT	480	520
FUN-1	5 μM	10 mM Na-HEPES, pH 7.2, 2% glucose	15	28	480	530-570
DAPI	1 μM	PBS	15	RT	340	410
MitoTrackerGreen	100 nM	10 mM Na-HEPES, pH 7.4, 5% glucose	15	RT	490	516
Rhodamine-Phalloidin	0.05 U/ml	PBS	30	RT	540	565

In situ detection of carbonylated protein

The carbonyl groups in the yeast cells were determined by reaction with 2,4-dinitrophenylhydrazine (DNPH) and detected with anti-DNP antibodies according to the protocol (**Aguilaniu *et al.*, 2003**). The protocol is based on the classical immuno-fluorescence protocols, we used 2,4-dinitrophenol-hydrazine to derivatize protein carbonyl groups and the primary polyclonal rabbit antibody against the dinitrophenyl-carbonyl moieties at a 1:10 000 dilution and the secondary goat anti-rabbit antibodies conjugated with Chromeo™ 546 at a 1:1000 dilution. For image acquisition OLYMPUS BX-63 fluorescence microscope was used, equipped with a DP-72 digital camera and analysis software cellSense.

Results and discussion

The yeast *S. cerevisiae* are very useful model for toxicity studies of various chemical compounds (**Bilinski *et al.*, 2005, Zheng *et al.*, 2007, Kwolek-Mirek *et al.*, 2009**). These studies are based mainly on assessment of cell viability. Traditional method of viability assay is the counting colony forming units on agar plates. This method has several limitations and it does not show various cellular parameters changes caused by the cytotoxic compounds (**Breeuwer and Abee, 2000**).

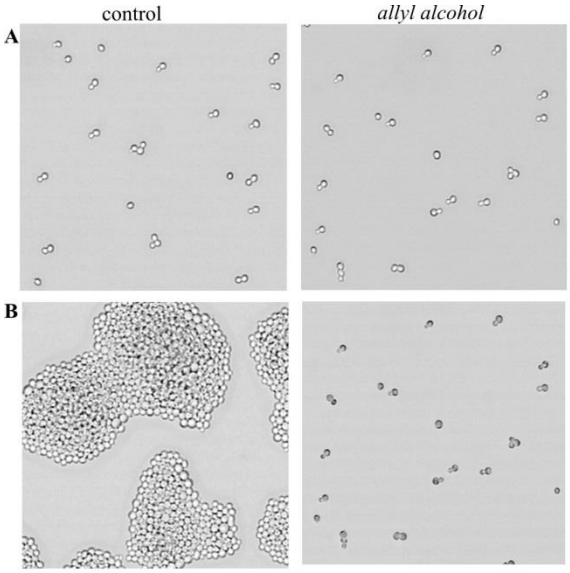

Figure 1
The effect of incubation with allyl alcohol on the yeast growth on solid medium. Cells growth were inspected within 24 h. (A) at the start of the experiment, (B) after 24 h of culture. *Magnification* 200×

Figure 1 shows a decrease in viability of the yeast cells treated with 0.4 mM allyl alcohol, which would indicate a very high toxicity of acrolein, which is produced by conversion of allyl alcohol inside the cell. This result does not allow to clearly state, that the cells which are arrested in the cell cycle are dead, or determine their metabolic state. The ability to divide is in fact the result of the many features as the metabolic activity of cells, the level of intracellular damage or intracellular organization.

Another recommended method to determinate cell viability are based on brightfield stains with a vital, unfluorescent dye PhloxineB (**Minois *et al.*, 2005**). This dye is absorbed by all yeast cells, but metabolically active cells are able to pump it out and remain colorless, and non-active or dead cells are red (Fig. 2A). However, the most interesting staining methods are the fluorescence techniques due to their higher sensitivity and specificity. The changes of the yeast cell viability could be detected using double staining method with fluoresceine diacetate (FDA) and propidium iodide (PI) (**Zheng *et al.*, 2007**). The non-fluorescent FDA molecules pass through the cell membranes and undergo hydrolysis by the cell esterases (**Breeuwer and Abee, 2000**). The reaction result is a fluorescent product – fluorescein, remains in the cytoplasm of vital cell (green fluorescent). Propidium iodide pass only through disrupted cell membrane and therefore PI positive cells (red fluorescent) are considered as dead cells (Fig. 2B).

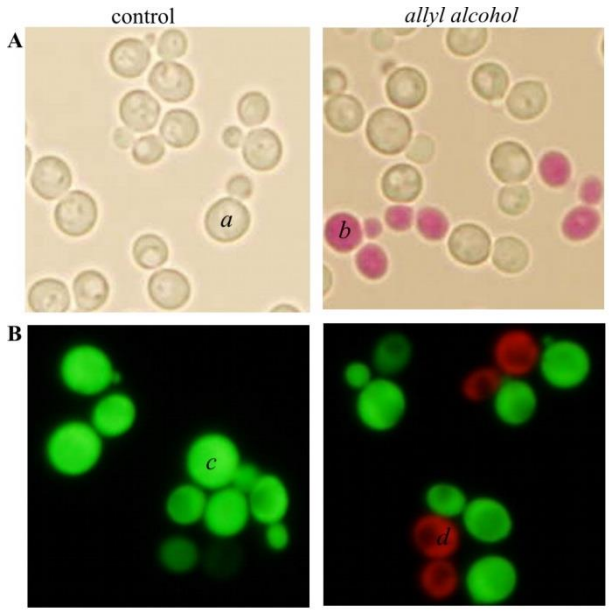

Figure 2
The effect of incubation with allyl alcohol on the yeast viability. (A) cells stained with PhloxineB, *a – live, colorless cell, b – dead, red cell*, (B) cells stained with FDA/PI *c – live, green fluorescent cell, d – dead (PI positive), red fluorescent cell. Magnification* 1000×

The percent of PhloxineB and PI positive cells is much higher for the cells exposed to acrolein and this parameter can be an indicator of the toxicity of this compound.

Both of these methods allow to determine the cell viability but some authors use this method as a test for determination also of the cell vitality as well (**Taya *et al.*, 1989**).

However, much more appropriate fluorophore for studying yeast cell vitality is the FUN-1 dye, which allow to distinguish between metabolically active and inactive or death cells (Fig. 3). This fluorescent, vital dye in metabolically active yeast cells is utilized and given the cylindrical intravacuolar structures within vacuoles (**Eggleston and Marshall, 2007**).

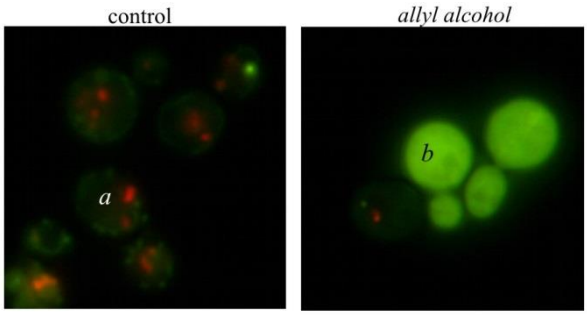

Figure 3
The effect of incubation with allyl alcohol on the metabolic activity of the yeast cells determined with FUN-1 fluorophore. *a – metabolically active cell with red, cylindrical structures within vacuole, b - metabolically inactive cell. Magnification* 1000×

The differences of the vitality and metabolic activity between untreated, controlled cells and cells treated with allyl alcohol, may be a consequence of the changes of cellular structure.

Using the specific for cellular organelles fluorophores as DAPI, Rhodamine-Phalloidin, MitoTrackerGreen can detect any changes in their morphology or localization within the cells (Fig.4). To assess the toxicity of acrolein or other chemicals the changes in the nucleus and DNA are particularly important. DAPI stained cells, after treatment with allyl alcohol, can show apoptotic phenotype as chromatin condensation or nucleus fragmentation (**Madeo *et al.*, 1999**). MitoTrackerGreen is green-fluorescent mitochondrial stain which appears to localize to mitochondria regardless of mitochondrial membrane potential while phalloidin tagged with the fluorescence dye - rhodamine is generally used to monitor distribution of actin microfilaments. The yeast actin cytoskeleton is an important structure that is organized into two structures. The first is actin cables which serve as a tracks for the movement of organelles including mitochondria or vacuoles and second is actin patches (**Pruyne *et al.*, 2004**). The actin microfilaments as highly dynamic structures and sensitive to various drugs and chemicals. Acrolein most likely reduces actin dynamic and it can lead to disintegration of mitochondrial network and a decrease in the cell viability. The changes of actin cytoskeleton are able to modulate also the level of reactive oxygen species (ROS) and consequently lead to increase in the level of oxidative damages (**Gourlay and Ayscough, 2005**).

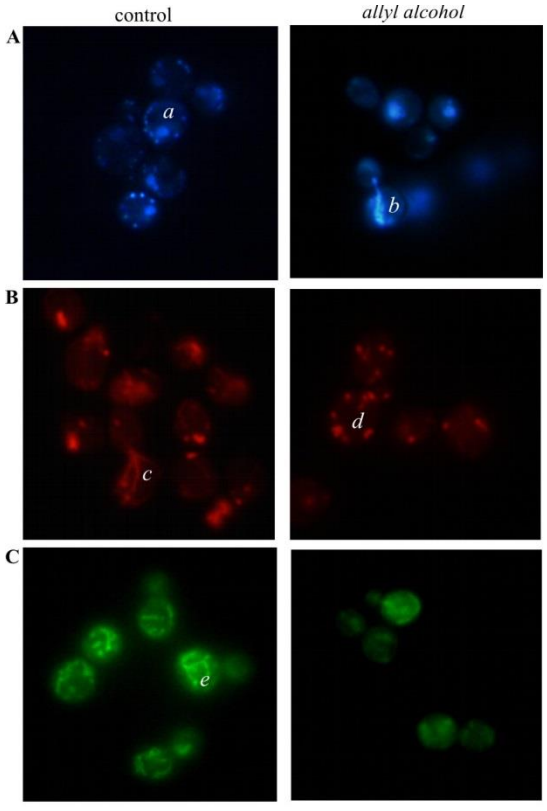

Figure 4
The effect of incubation with allyl alcohol on the cellular organelles. (A) yeast nucleus stained with DAPI, *a – proper nucleus, b - nucleus fragmentation;* (B) yeast actin cytoskeleton stained with Rhodamine-Phalloidin, *c – actin cables, d – actin patches;* (C) yeast mitochondria stained with MitoTrackerGreen, *e – mitochondrial network*. *Magnification* 1000×

To visualization and localization one of the types oxidative damages – oxidative protein damage, indirect immunofluorescence microscopy is the most useful procedure.

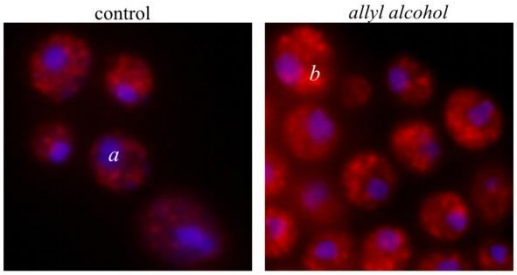

Figure 5
Distribution of oxidatively damaged proteins in yeast cells treated with allyl alcohol,
a – nucleus, b – carbonyl proteins.
Magnification 1000×

This result clearly shows that acrolein leads to increase in the level of the oxidative protein damage within the cells.

All analyzes were performed on both the wild-type strain and Δ*sod1* yeast strain deficient in Cu,Zn-superoxide dismutase, one of the most important enzymes for the cell antioxidant defense. Presented results are based, however, only on the Δ*sod1* yeast cells because these cells are hypersensitive to acrolein, thus observed changes are significantly more pronounced than in wild-type cells and therefore better highlights the advantages of presented methods. This confirms that the mechanism of acrolein toxicity involves induction of oxidative stress in the cell.

Conclusion

The analysis of a various parameters of the cells exposed to acrolein, indicates that the methods based on fluorescence microscopy are a very useful and helpful research tool to assess toxicity of various chemicals. An important advantage is that it is a relatively fast way allowing to determine viability, vitality and metabolic activity of the cells, changes of cellular organelles morphology or intracellular distribution macromolecules damage. These parameters may be therefore useful in identifying the toxicity mechanism of various chemicals. Moreover this fluorescent microscopy method has potential application for high throughput screening for novel drugs or food ingredients.

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