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# TRIAGEM DE CEPAS DE *Saccharomyces cerevisiae* SENSÍVEIS AO METILGLIOXAL

**DISSERTAÇÃO DE MESTRADO**

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**Santa Maria, RS, Brasil**
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# TRIAGEM DE CEPAS DE *Saccharomyces cerevisiae* SENSÍVEIS AO METILGLIOXAL

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS),
como requisito parcial para obtenção do grau de
**Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

Orientadora: Profª. Drª Nilda Berenice de Vargas Barbosa

Co-orientadora: Profª. Drª Cristiane Lenz Dalla Corte

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**TRIAGEM DE CEPAS DE *Saccharomyces cerevisiae* SENSÍVEIS AO METILGLIOXAL**

Elaborada por

**Sandra Sartoretto Pavin**

Como requisito básico para a obtenção do grau de
**Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

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**Santa Maria, 15 de Janeiro de 2015.**

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## MANUSCRITO

## LISTA DAS ABREVIATURAS

**MG:** Metilglioxal.

**AGEs:** Produtos Finais de Glicação Avançada.

**RAGEs**: Receptores de produtos de Glicação Avançada.

**GSH:** Glutationa.

**ROS:** Espécies reativas de oxigênio.

***S. cerevisiae:*** *Saccharomyces cerevisiae.*

**IC:** Concentração inibitória.

**YPDGal:** Meio de cultura com galactose.

**YPDGlu:** Meio de cultura com glicose

**AMP:** Ampicilina

**RESUMO**

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

# Triagem de cepas de *Saccharomyces cerevisiae* sensíveis ao metilglioxal

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LOCAL E DATA DA DEFESA: Santa Maria, 15 de janeiro de 2015.

O metilglioxal (MG) é um composto α-dicarbonílico, muito reativo, que pode ser formado em grandes quantidades sob condições hiperglicêmicas. Ele é produzido principalmente por vias não enzimáticas a partir de intermediários glicolíticos como o gliceraldeído-3-fosfato e a di-hidroxiacetona fosfato. Esse composto é conhecido por reagir com macromoléculas tais como proteínas, DNA e RNA, alterando suas funções. No entanto, os mecanismos pelos quais o composto induz toxicidade nos sistemas biológicos não se encontram totalmente elucidados. Assim, o presente trabalho teve como objetivo realizar uma triagem para identificar cepas mutantes de *Saccharomyces cerevisiae* (*S. cerevisiae*) sensíveis ao MG; usando principalmente mutantes com genes suprimidos para o estresse oxidativo e danos ao DNA. Noventa e seis cepas mutantes foram colocadas em meio YPD-galactose contendo diferentes concentrações de MG (5 mM a 12 mM). A sensibilidade ao MG foi avaliada através de parâmetros de crescimento e viabilidade celular. Os diferentes testes mostraram que a toxicidade do MG foi mais pronunciada em cepas com deleção em genes envolvidos com eventos de reparo ao DNA: Rad23 e Rad50. A exposição ao MG também diminuiu significativamente o crescimento e a viabilidade celular das cepas mutantes com genes deletados para a enzima glioxalase1 (Glo1) e glutationa (Gsh1). Os resultados obtidos destacam a importância do sistema glioxalase1 na detoxificação do MG em *S. cerevisiae* e apontam o DNA como molécula alvo à toxicidade do composto.


**Palavras chaves:** Metilglioxal, *Saccharomices cerevisiae, DNA, estresse oxidativo.*

## ABSTRACT

Dissertation of Master's Degree
Graduate Course in Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

**Screening of *S. cerevisiae* strains sentitive to methylglyoxal**

AUTHOR: Sandra SartorettoPavin
ADVISOR: Nilda Berenice de Vargas Barbosa
CO-ADVISOR: Cristiane Lenz Dalla Corte
PLACE AND DATE OF THE DEFENSE: Santa Maria, 15th January, 2015.

Methylglyoxal is a very reactive α-dicarbonilic compound, that can can be formed in high concentrations in hyperglycemic conditions. It is produced mainly via non-enzymatic ways from glycolytic intermediates, like glyceraldehyde-3-phophate and di-hydroxyketone phosphate. This compound is known for reacting with macromolecules such as proteins, DNA, and RNA, altering their funtions. However, the toxic mechanisms involved in MG toxicity on the biological systems are not fully elucidated. Thus, the present study aimed to perform a screening to identify *Saccharomyces cerevisiae* mutant strains sensitive to MG, using mainly strains with oxidative stress and DNA damage related mutations. Ninety-six mutant strains were placed in YPD-galactose medium containing different MG concentrations (5-12 mM). The MG sensitivity was evaluated through growth and cellular viability parameters. The different tests showed that the MG toxicity was more pronounced in strains with deletions in genes involved with DNA repair events: Rad23 and Rad50. The MG exposure also decreased markedly the growth and cellular viability in the mutant strains with deletions in genes for the enzymes glyoxalase 1 (Glo1) and glutathione (Gsh1). The results obtained highlight the importance of the glyoxalase 1 system in MG detoxification and point the DNA as a target for the toxicity of the compound in *S. cerevisae*.


**Key words:** methylglyoxal, *S. cerevisiae*, screening, oxidative stress.

# APRESENTAÇÃO

No item **INTRODUÇÃO**, está descrito uma sucinta revisão bibliográfica sobre os temas abordados nesta dissertação.

O **DESENVOLVIMENTO** está apresentado sob a forma de um manuscrito, o qual se encontra alocado no item **MANUSCRITO**.

As seções Materiais e Métodos, Resultados e Discussão encontram-se no próprio manuscrito e representam a íntegra deste estudo.

O item **CONCLUSÕES** e **PERSPECTIVAS** são apresentados no final desta dissertação e apresentam interpretações e comentários gerais sobre a investigação desenvolvida.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO**, uma vez que o manuscrito contém as suas próprias referências.

## 1.INTRODUÇÃO

O metilglioxal (MG) é um composto α-dicarbonílico, muito reativo, que pode ser formado sob condições hiperglicêmicas. Indivíduos diabéticos usualmente apresentam níveis elevados de MG no plasma e tecidos (Brownlee, 1995), os quais têm sido associados com aumento do risco de complicações diabéticas tais como nefropatia, retinopatia, neuropatia e complicações cardiovasculares (Inoueet al., 1995).

O MG é produzido principalmente por vias não enzimáticas a partir de intermediários glicolíticos como o gliceraldeído-3-fosfato e a di-hidroxiacetona fosfato (O'Brien et al., 2010). Outras rotas incluindo o metabolismo de proteínas e ácidos graxos, a peroxidação lipídica e a degradação do DNA são consideradas importantes fontes de MG (Chaplenet al., 1996; Zou et al., 2012; Guo et al., 2013; Li et al., 2013; Wu et al., 2013). Em condições hiperglicêmicas, destacam-se a reações de glicação não-enzimáticas, onde os grupos carbonila de carboidratos reagem com os grupamentos amino de proteínas formando inicialmente bases de *Schiff*. Estas, por sua vez, sofrem rearranjos espontâneos formando os chamados produtos de Amadori, denominados também de produtos iniciais de glicação (Yeboahet al., 1999). Posteriormente, os produtos de Amadori podem ser degradados e formar compostos α-dicarbonílicos tais como o glioxal, o 3-deoxiglucosona e o MG, cujos grupamentos carbonila são ainda mais reativos frente aos grupos amino de proteínas. Como resultado de tal interação, ocorre a formação dos produtos finais de glicação avançada (AGEs) (Onorato et al., 1998 ) (Figura1).

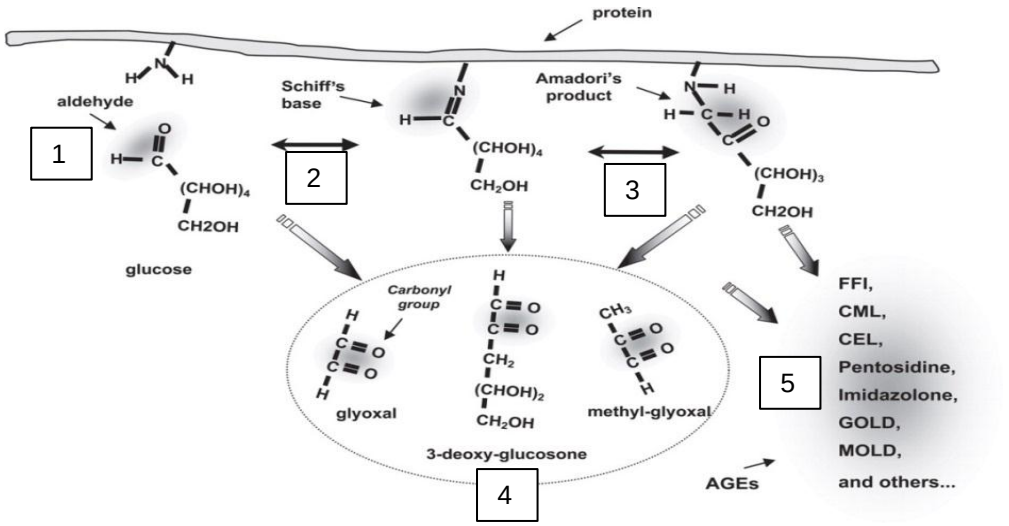

Figura 1: Formação de Produtos de Glicação (Basta et al., 2004). (1) Reação de grupos carbonila de carboidratos com grupamentos amino de proteínas; (2) formação de bases de *Schiff*; (3) produtos de Amadori; (4) compostos α-dicarbonílicos e (5) produtos finais de glicação avançada (AGEs).

Além de proteínas, estudos têm evidenciado que os efeitos adversos do MG em nível celular são causados pela capacidade de modificar a estrutura de nucleotídeos. O composto é considerado altamente genotóxico e mutagênico por reagir com resíduos de guaninado DNA (Shapiro et al., 1969; Kalapos, 2008). Murata e colaboradores mostraram que o acúmulo celular de MG causa tanto efeitos citostáticos quanto citotóxicos (Murataet al., 1985).

Alguns estudos têm demonstrado uma estreita relação entre os produtos de glicação e o estresse oxidativo. Os AGEs, formados a partir das reações de glicação envolvendo o MG, são conhecidos por estarem envolvidos em diversos eventos celulares associados com a patologia de doenças como o diabetes mellitus, aterosclerose, doença de Alzheimer e envelhecimento (Bucala et al., 1992; Sato et al., 2006; Gomes et al., 2005). Os AGEs têm receptores (RAGEs) em diversos tipos celulares, onde desencadeiam dano oxidativo via formação de espécies reativas de oxigênio (ROS) (Martins et al., 2001; Wang et al., 2014;Feng et al., 2014). Além disso, o MG *per se* pode causar estresse oxidativo pela redução nos níveis de glutationa (GSH), uma vez que a detoxificação do mesmo culmina com a depleção de GSH. O MG é catabolizado principalmente por duas vias enzimáticas (Figura 2), cuja importância fisiológica ainda é em grande parte desconhecida (Bitoet al., 1997). Este sistema de detoxificação do MG é denominado sistema glioxalase, e compreende a ação das enzimas glioxalase I e glioxalase II. A glioxalase I catalisa a conversão do MG à D-lactoilglutationa depletando GSH. O éster de tiol da glutationa formado é então hidrolisado pela glioxalase II à lactato. Estas enzimas foram inicialmente descobertas em tecidos de coelhos e cães e relatadas pela primeira vez há cerca de 100 anos (Thornalley, 1993). Particularmente, a glioxalase I é reconhecida por sua natureza ubíqua.

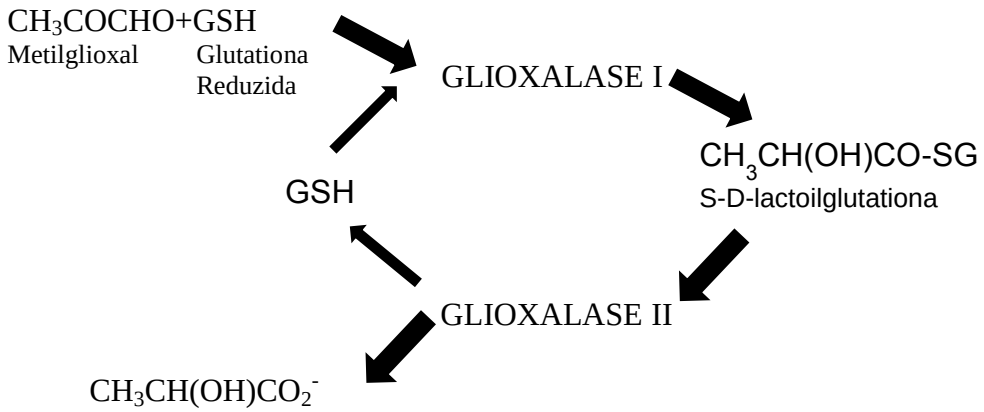


**Figura 2.** Sistem

Embora os processos de glicação sejam conhecidos por seus efeitos deletérios às células, os mecanismos envolvidos na citotoxicidade mediada pelo MG, um dos maiores precursores dos AGEs, ainda não se encontram totalmente elucidados. Neste contexto, o uso de modelos alternativos para estudos mecanicísticos vem crescendo muito nas últimas décadas e várias pesquisas voltadas para estudar aspectos metabólicos e toxicológicos do MG e AGEs estão sendo feitas usando a levedura *Saccharomyces cerevisiae* (*S. cerevisiae*) como organismo alvo (Martins et al., 2001; Penninckx et al., 1983). Na *S. cerevisiae* ~ 0,3% de todo o fluxo de carbono da via glicolítica é convertido em MG, tornando-o um metabolito potencialmente abundante em condições de crescimento que favoreçam elevadas taxas de glicose (Wendleretal., 2009). A concentração endógena de MG em *S. cerevisiae* é igual a 31μM ou 2,5 pmol/ $10^6$ células (Maetaet al., 2005).

### 1.1. *Saccharomycescerevisiae* como modelo experimental

A levedura *S. cerevisiae* foi o primeiro eucarioto a ter seu genoma completamente sequenciado (Goffeauet al., 1996).Atualmente, o *Saccharomyces*GenomeDatabase (SGD: http: //www.yeastgenome.org/) fornece informações sobre cada gene deste organismo.As vantagens apresentadas para o uso de *S. cerevisiae* como organismo modelo são o genoma pequeno (aproximadamente 200 vezes menor que o humano), a multiplicação rápida (cerca de 2 horas) e a facilidade de manipulação pela morfologia da célula e do núcleo (Henriques et al., 1997; Barr, 2003; Botstein et al., 2011). Além disso, as leveduras apresentam estágio haplóide e diplóide durante o ciclo de vida, permitindo a investigação de mutações recessivas que podem ser mascaradas pelo alelo selvagem no estado diplóide (Perego et al., 2000).

Entre os benefícios apresentados pela levedura *S. cerevisiae* para fins de pesquisa, a facilidade da combinação de mutações foi uma das principais razões que despertou o interesse entre os pesquisadores para seu uso como organismo modelo no final da década de 1980 (Botstein et al., 1988). Embora muitas vezes estudos envolvendo a expressão de proteínas heterólogas em leveduras sejam vistos com ceticismo devido aos níveis de expressão não fisiológica (Pereira et al., 2012), a reconhecida contribuição destes eucariotos unicelulares para o atual conhecimento de aspectos fundamentais da biologia celular de eucariotos superiores justifica sua exploração como modelo de sistema celular (Matuo et al., 2012; Pereira et al, 2012).

O uso da levedura como modelo de célula experimental tem auxiliado no conhecimento e no tratamento de uma série de patologias humanas devido à conservação de

mecanismos bioquímicos que são observados nas células humanas (Bertolinet al., 2009; Outeiro et al., 2007; Lindquist, 2003).

Além disso, a levedura é um modelo de pesquisa cuja utilização pode elucidar muitos aspectos importantes nas patologias humanas que são mais difíceis de estudar usando outros organismos modelo eucariotos mais complexos (Farrugia et al, 2012).

## 2. OBJETIVOS

### *2.1 Objetivo Geral*

Através de uma triagem usando cepas mutantes de *S. cerevisiae*, identificar alvos moleculares sensíveis ao MG.

### *2.2 Objetivos Específicos*

Através da análise do crescimento celular:

- comparar a sensibilidade de leveduras selvagem ao MG em meios de cultura contendo glicose ou galactose.

- realizar uma "triagem" de cepas mutantes para genes envolvidos com o estresse oxidativo e reparo no DNA sensíveis ao MG em meio sólido e líquido.

- selecionar as cepas mutantes sensíveis ao MG e verificar a $CI_{50}$ do MG para as mesmas.

Analisar a viabilidade celular das cepas selvagens e mutantes expostas ao MG.

## 3. DESENVOLVIMENTO

O desenvolvimento referente a essa dissertação está apresentado sob a forma de manuscrito. Os itens Materiais e Métodos, Resultados, Discussão e Referências encontram-se no próprio manuscrito.

MANUSCRITO: Triagem de cepas de *S. cerevisiae* mutantes destaca proteínas de reparo de DNA e glyoxase I como alvos para toxicidade do metilglioxal.

**Screening of *S. cerevisiae* mutant strains highlights DNA repair checkpoint and glyoxase I proteins as targets for methylglyoxal toxicity.**

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**Abstract**

Methylglyoxal (MG) is a toxic compound known by forming covalent adducts with macromolecules such as protein, DNA and RNA, potentially disrupting cellular functions. However, the phenomena triggered by MG in eukaryotic cells are until little known. In this study, we performed a screening to identify *S. cerevisiae* mutant strains sensible to MG, emphasizing mutants with genes deleted for oxidative stress and DNA repair. The mutant strains were placed in YPD-Galactose medium containing different concentrations of MG (5 to 12mM). The sensitivity or resistant mutant strains to MG was evaluated through growth cellular and cell viability. The different screenings showed that MG toxicity was more pronounced in strains with deletion in genes involved with DNA repair checkpoint proteins, namely Rad23 and Rad50.Likewise, MG impaired the growth and cell viability of *S. cerevisiae* mutant strains Glo1 (Glutathione metabolic process/ methylglyoxal catabolic process to D-lactate) and Gsh1 (Glutamate-cysteine ligase activity), which comprise the glyoxalase I system. In summary, our results highlight the crucial role of glyoxalase I pathway in the detoxification of MG as well as point the DNA as target molecule for MG toxicity in *S. cerevisiae*.

**Key words**: methylglyoxal, *S. cerevisiae, screening, oxidative stress*

## 1. Introduction

Methylglyoxal(MG), a physiological dicarbonyl metabolite, is known to be toxic to different organisms due to its high reactivity. It is produced primarily by non enzymatic pathways from glycolytic intermediates such as glyceraldehyde 3-phosphate and dihydroxyacetone phosphate as well as by several minor metabolic pathways, including the Maillard reaction; protein, fatty acid and acetone metabolism and DNA degradation (Thornalley, 1993; Chaplen et al., 1996; Zou et al., 2012; Guo et al., 2013; Li et al., 2013; Wu et al., 2013). In microorganisms the aldehyde is mainly generated from dihydroxyacetone phosphate by MG synthase (Kalapos, 1999).

Clinical and experimental data have shown that MG is frequently found at high levels in blood and tissues of diabetics and that its toxicity is mediated by reaction with macromolecules such as proteins, nucleotides and lipids (Eguchi et al., 2013; Liu et al., 2013). The aldehyde structure of MG may react nonenzymatically with arginine, lysine and sulfhydryl groups in proteins, inactivating them (Murata et al., 1985). Indeed, MG elicits genotoxic and mutagenic effects through the modification of guanyl residues of DNA (Shapiro et al., 1969). These unstable intermediates generated from glycation by MG are precursors of the advanced glycation end products (AGEs), which are known to play a central role in the pathogenesis of diabetic complications and other diseases as atherosclerosis, renal failure, Alzheimer's disease and ageing (Bucala et al., 1992; Sato et al., 2006; Gomes et al, 2005).

Several studies have demonstrated a strong relationship between glycation and oxidative events in different model systems (Brownlee, 2000; Bourajjaj et al., 2003; Semchyshyn 2013). Under oxidative stress, there is an elevation in the MG levels due GSH depletion, which may cause progressive tissue dysfunctions (Masterjohn et al., 2013). This effect can be further magnified in a positive feedback loop because AGEs themselves contribute to the production of reactive oxygen species (Martins et al., 2001).

Although, MG has been found to disrupt cellular function from microorganisms to mammals, the metabolism aspects of MG in eukaryotic cells is little known (Ahmed et al., 1986; Egyud et al, 1966; Fodor et al., 1967). In this scenario, the yeast *S. cerevisiae* has emerged as an advantageous organism to study the biochemistry of MG metabolism and AGEs formation (Martins et al., 2001; Penninckx et al., 1983). In *S. cerevisiae* ~0.3% of all glycolytic carbon flux is converted to MG, making it a potentially abundant metabolite under growth conditions that favor high rates of glycolysis (Wendler et al., 2009). Important, genes encoding enzymes for MG detoxification have already been identified and characterized in

the yeast (Inoue et al., 1996; Bito et al., 1997). Moreover, *S. cerevisiae* is considered an important alternative model to vertebrates due its well documented metabolic, growth and genetic molecular machinery (Botstein et al., 1988.)

Considering the importance of more detailed information about the harmful effects elicited by MG and the advantages of *S. cerevisiae* for investigation of many mammalian aspects, the present study was delineated to identify *S. cerevisiae* mutant strains sensible to MG, emphasizing oxidative stress and DNA damage. This screening will facilitate the selection of strains more adequate for the study of MG cytotoxicity.

## 2. Materials and Methods

### *2.1 Reagents and yeast strains*

Agar, bactopeptone and yeast extract were purchased from Difco (USA). MG was purchased from Sigma (USA). The other chemicals were of analytical grade and purchased from local commercial suppliers. All experiments were performed with the wild type BY4741 (MATa: his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) and single deletion mutants, which were kindly donated by Mônica Lomeli e Cláudio Masuda from UFRJ-Brazil. The *S. cerevisiae* single deletion mutants used in this work are listed in Table 1.

### *2.2 Growth curve and medium selection assay*

Firstly, the growth curves were performed with YPD-Galactose and YPD-Glucose (YPDGal and YPDGlu) and different concentrations of MG, in order to select the medium and the inhibitory concentrations of MG for subsequent tests. The wild type BY4741 strain was growth on YPDGal (2% peptone, 1% bacto yeast extract, 2% galactose) and YPDGlu (2% peptone, 1% bacto yeast extract, 2% glucose) liquid medium supplemented with 100mg/ml AMP till log phase, diluted 1000 times, and treated with MG at concentrations of 0.5, 1, 2.5, 5. 7.5 and 10mM. Yeast cells were placed to grow for 24 hours, at 30°C under shaking at 200 rpm. Afterwards, the cell density was determined by measuring the absorbance at 600 nm. The subsequent experiments were performed in YPDGal, due the higher sensitivity of yeasts to MG in this medium.

### *2.3 Screening of S. cerevisiae mutant strains sensitive to MG*

The first screening of MG sensitivity *S. cerevisiae* mutants (from MATa his3D1 leu2D0 met15D0 ura3D0) was performed using the strains listed in the Table 1. Briefly, the individual deletion strains present in 96-well master plate were resuspended with a multichannel pipette, replicated using a replica plater and spoted in YPGal agar medium containing different concentrations of MG (5, 7.5, 10 and 12.5mM). Afterwards, the plates were incubated for 48 hours at 30ºC and the resulting plates were imaged using a Canon camera. The MG sensitivity mutant strains were further analyzed. Briefly, the wild type strain and the selected MG sensitivity mutants were inoculated into YPDGal liquid medium and incubated at 30°C and 200 rpm until saturation. The yeast saturated cultures were serially diluted ($10^{-1}$, $10^{-2}$ , $10^{-3}$) in 300µl of sterile distilled water, and 3 µl spotted into YPDGal

agar plates containing different concentrations of MG (5, 7.5, 10 and 12.5mM). Afterwards, the plates were incubated for 48 hours at 30ºC and the resulting plates were imaged using a Canon camera. The mutants were then classified as sensitive or resistant to MG compared to the wild type strain (BY4741). All experiments were done in triplicate.

*2.4 Growth curve of MG sensitivy S. cerevisiae mutant strains in liquid medium.*

The mutants whose gene deletion caused sensitivity to MG were growth on YPDGal liquid medium until the log phase, diluted 1000 times. Afterwards, the cells were treated with MG at different concentrations (0.5, 1, 2.5, 5, 7.5 and 10mM) for 24 hours at 30°C and 200 rpm. The density was determined by measuring the absorbance at 600 nm. The concentration of MG that caused half-maximal inhibition of growth ($IC_{50}$) was calculated using GraphPad Prism 5 program.

*2.5 Evaluation of cell viability*

To assess the cell viability, the mutant strains whose gene deletion caused sensitivity to MG were growth on YPDGal liquid medium until log phase, diluted 1000 times, and treated with MG at different concentrations (0.5, 1, 2.5, 5, 7.5 and 10mM). The cells were placed to grow for 24 hours, at 30°C and 200 rpm. After the exposure to MG, the cells were serially diluted and plated onto solid YPGal medium. The cultures were then incubated at 30ºC for 72 h and the cell viability evaluated.

2.6 Statisical analyzes

Data are expressed as means ± SD. Statistical analysis was performed using One-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison test when appropriate. Differences were considered significant when $P < 0.05$.

## 3. Results

### *3.1 Growth of wild type yeast exposed to MG in liquid medium containing glucose and galactose*

Firstly, we performed a growth curve with wild type yeast in galactose and glucose liquid medium for 24 hours, in order to choose the medium that could confer more sensitivity of the yeast to MG. The results of figure 1 show that the wild type yeasts were more sensitive to MG when grown in the presence of galactose than glucose. The $IC_{50}$ found to MG on the yeast growth in the presence of galactose and glucose was 1,247 and 4,325, respectively (data not shown). Thus, the further experiments were performed with yeast grown in galactose.

### *3.2 Growth of yeast mutant strains exposed to MG in solid medium.*

Firstly we screened a library of 96 *S. cerevisiae* gene-deletion mutants, in order to identify the strains resistant and/or sensitive to MG. Among them, 30 mutant strains were identified as sensitive and selected for a secondary screening, where the sensitive of the yeasts was confirmed using three different dilutions. The results obtained of this screening show that *S. cerevisiae* mutant strains related to DNA repair checkpoints as Rad23, Rad50 and Stb5 were more sensitive to MG when compared the others (figure 2). Likewise, the mutant strains Gsh1 and Glo1 presented high sensitivity to MG. In general, the toxic effect of MG was observed from 7.5mM. The sensitivity of these five mutants was subsequently confirmed through a growth curve in liquid medium.

### *3.3 Growth of yeast mutant strains exposed to MG in liquid medium*

The Figure 3 shows the effect of MG exposure on growth of mutant strains in liquid medium. In this assay, MG exposure affected mainly the growth of mutant strainsRad50, Rad23, Gsh1 and Glo1 when compared to the wild type strain (Figure 3A, 3B, 3D and 3E). Unlike, the mutant strain Stb5 was more resistant to MG when compared to the wild type strain (Figure 3C). The $IC_{50}$ found for the different mutants strains exposed to MG was in the following order: Glo1 < Gsh1< Rad 23< Rad 50< Stb5 (Table 2).

*3.4 Cell viability of mutant strains Glo1, Gsh1, Rad 23, Rad 50 and Stb5 exposed to MG.*

The figure 4 shows that cellular viability of mutant strains Glo1, Gsh 1, Rad23 and Rad50 was potentially disrupted by MG when compared to the wild type strain (Figure 4A, 4B, 4D, 4E). The sensitivity of mutants to MG was in the following order: Glo1 > Rad 23 >Gsh1>Rad 50. Different, the mutant strain Stb5 was resistant to MG exposure (Figure 4F).

## 4. Discussion

It has been reported that MG accumulates under conditions of hyperglycemia, impaired glucose metabolism and oxidative stress (McLellan et al., 1994, Liu et al., 2012; Nenov, 2014; Sena et al., 2012) and that its toxicity is mainly linked to glycation events, which may culminate with AGEs formation and ROS overproduction (Brownlee, 2000; Bourajjaj et al., 2003; Savu et al., 2011; Giacco et al., 2010). With emphasis in oxidative stress and DNA damage, in this study we used *S. cerevisae* mutant strains as alternative model to identify target proteins linked to MG toxicity. We show here that the set of yeast mutant strains sensitive to MG had deletion in genes engaged with DNA repair and MG detoxification. MG exposure caused inhibition of growth and loss of cell viability specifically in the strains Rad23, Rad50, Glo1 and Gsh1.

The pathways for MG detoxification have been extensively studied in many organisms (Inoue et al., 1995; Kalapos, 1999). In yeast, the catabolism of MG may be performed by glyoxylase system, MG reductase and aldose reductase enzymes (Marmstal et al., 1979; Inoue et al., 1996; Aguilera et al., 2004; Gomes et al., 2005). Among them, the glyoxalase system has been thought to be the major detoxifying route for MG. The system comprises the enzyme glyoxalase I (encoded by the Glo1 gene) that converts MG to S-D-lactoylglutathione using glutathione as specific cofactor; and the enzyme glyoxalase II (encoded by the Glo2 and Glo4 genes) that hydrolyses this glutathione thiolester to D-lactic acid (Bito et al., 1997; Aguilera et al., 2004; Inoue et al. 2011). Here we analyzed the effects of MG on this system, particularly in strains lacking Glo1, Glo2 and Gsh genes. We found that MG inhibited the growth and decreased the cell viability of *S. cerevisiae* Glo1 and Gsh1 mutant strains. On the order hand, these parameters were not disrupted in Glo2 mutants. Taken together, these findings give a particular indication for a regulatory role of glyoxalase I pathway in response to MG in *S. cerevisiae.* In this way, there is evidence that carbonyl compounds as glycerol and MG provoke growth arrest and cell killing in *S. cerevisiae* glyoxalase I deficient mutant strains (Penninckx et al., 1983; Inoue et al.,1999; Takatsume et al., 2004). In addition, it has been shown that MG is able to activate the expression of Glo1 and Gre3, two genes involved in MG detoxification and GPD1, gene for glycerol synthesis (Aguilera et al., 2004).

Many studies have demonstrated the potential genotoxic of MG in human cells (Sharma et al., 2014; Schupp et al., 2005). MG is known by inducing both DNA–DNA and DNA-protein crosslink, being considered more potent crosslinking reagent than other reactive carbonyl aldehydes, such as glyoxal and formaldehyde (Roberts et al., 2003; Brambilla et al.,

1985; Murata-Kamiya et al., 2001). As a result of their bulky nature and their ability to distort DNA structure, these DNA adducts are expected to alter crucial cellular processes, including DNA replication, transcription, repair, recombination, and chromatin remodeling, thus potentially contributing to spontaneous mutagenesis, cytotoxicity and aging (Grillari et al., 2007; Barker et al, 2005). In this sense, there are findings showing that MG can crosslink guanine residues of DNA as well as lysine and cysteine residues of enzyme DNA polymerase, causing inhibition of DNA synthesis (Hou et al., 1995; Wu et al., 2007; Kang et al., 2003; Murata-Kamiya et al., 2001). Evidence also suggests that AGEs products may accumulate in nuclear proteins like histone causing extensive DNA strand cleavage (Roberts et al., 2003).

Literature data support that ROS formation may contribute to the DNA damage elicited by MG. In the ROS dependent pathway, the production of superoxide anion during the glycation reaction of MG with the lysine residues of amino acids could lead to subsequent production of hydroxyl radicals, which is thought cause DNA cleavage (Babizhayev et al., 2014). Supporting the participation of oxidative phenomena in MG-induced cell injury, some *in vitro* studies have demonstrated the efficacy of antioxidants in blocking ROS generation, oxidative DNA damage and apoptosis induced by MG (Wu et al., 2007; Huang et al., 2008). Although the genotoxic and oxidative effects of MG is relatively well documented in mammalian, in yeast it is poor understood. However, related studies have demonstrated that yeast strains with different defects in the antioxidant defense are sensible to glyoxal and that the MG is able to activate transcription factors linked to oxidative stress pathways (Maeta et al., 2004; Takatsume et al., 2006; Semchyshyn, 2013). Here, we performed a screening with *S. cerevisiae* mutant strains linked to the antioxidant/oxidative machinery, in order to identify target proteins for MG toxicity. In general, the strains with deletion in genes related to the antioxidant defenses such as superoxide dismutase, catalase, glutathione peroxidase and thioredoxinreductase enzymes were resistant to MG. Moreover, we found that the mutant strain with deletion in the Yap1 gene, which is critical for the oxidative-stress response in *S. cerevisiae* was not sensitivity to MG.

In our experimental protocol, MG toxicity was more pronounced in strains with deletion in genes involved with DNA repair checkpoints, namely Rad23 and Rad50. The protein encoded by Rad23 gene contains an N-terminal ubiquitin-like domain and is involved with nucleotide excision repair processes while Rad50 is a component of the MRN complex, which plays a central role in double-strand break repair, chromatids recombinational behavior and meiosis (Liefshitz et al., 1995; Malkova et al., 1996; Lambert et al., 2003; Xie et al., 2004; Watkins et al., 1993).

In summary, the screening performed with *S. cerevisiae* mutant strains led to the identification of genes important to understanding the molecular mechanisms involved in the MG cytotoxicity. Here we confirm the crucial role of glioxalase I pathway in the detoxification of MG as well as point the DNA as target molecule for MG toxicity in *S. cerevisiae*. Probably the stress oxidative had a middle participation on the harmful effects elicited by MG in the growth and cell viability of mutant strains, since most of mutants with genes deleted to antioxidant defenses were not affected by MG.

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**Figure 1**

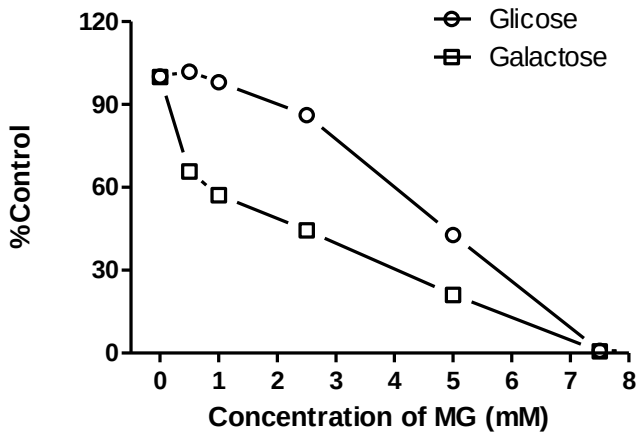


**Figure 1. Effect of MG exposure on the growth of wild type yeast in liquid medium**. Wild type yeasts were incubated with different concentrations of MG (0.5; 1; 2.5; 5; 7.5mM) for 24 hours in YPD-Glu or YPD-Gal liquid medium.

## Figure 2

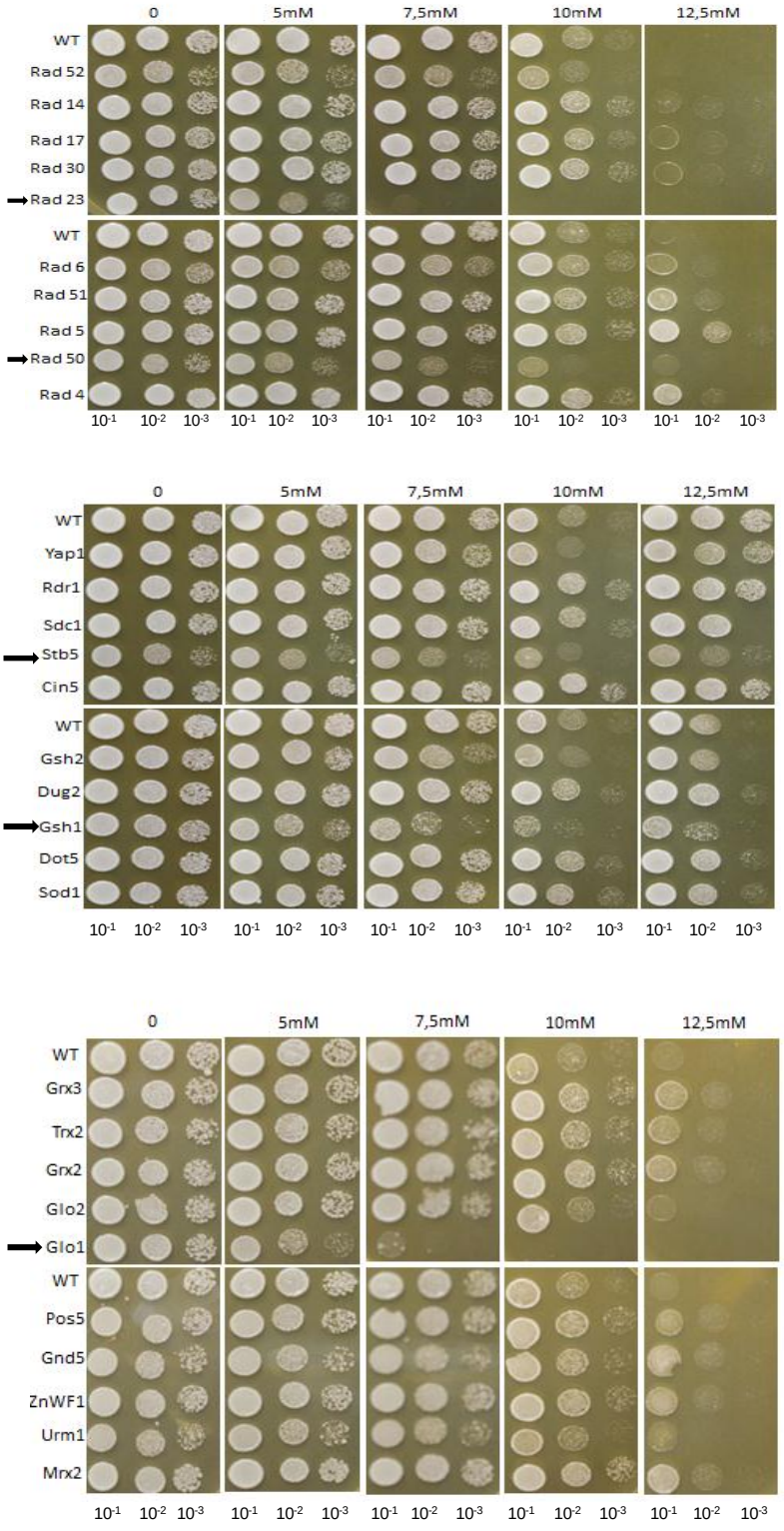


**Figure 2. Effect of MG exposure on the growth of yeast mutant strains in solid medium.** The saturated yeast cultures were serially diluted ($10^{-1}$, $10^{-2}$, $10^{-3}$) in 300µL of sterile distilled water and then spotted onto plates containing YPD-Gal agar and differentconcentrations of MG (5, 7.5, 10 and 12.5 mM) for 24 hours (n=3).

**Figure 3**

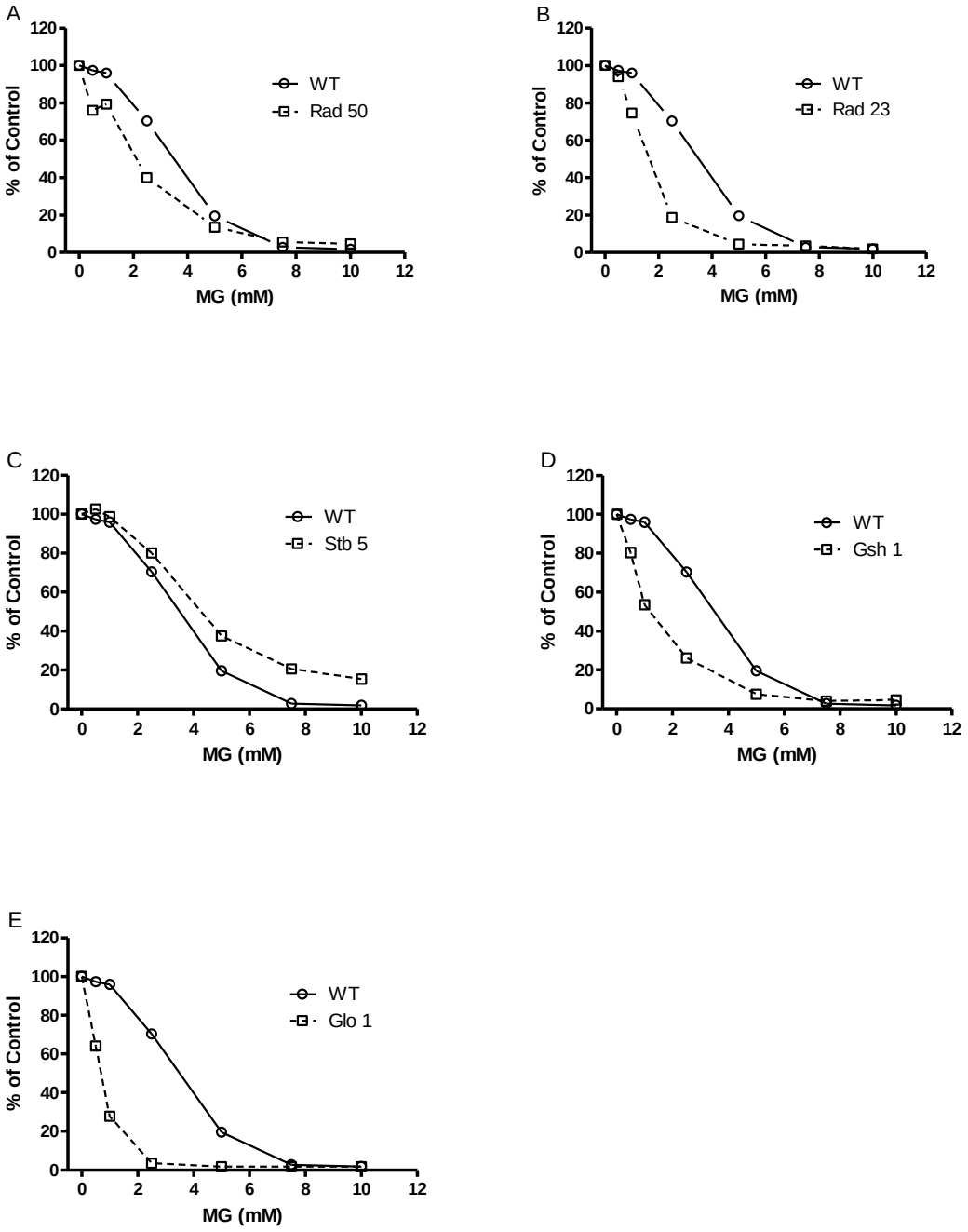


**Figure 3.Effect of MG exposure on the grown of yeast mutant strains in liquid medium**. Mutant and wild type strains were incubated with different concentrations of MG (0.5, 1, 2.5, 5, 7.5 and 10 mM) for 24 hours(n=4).

**Figure 4**

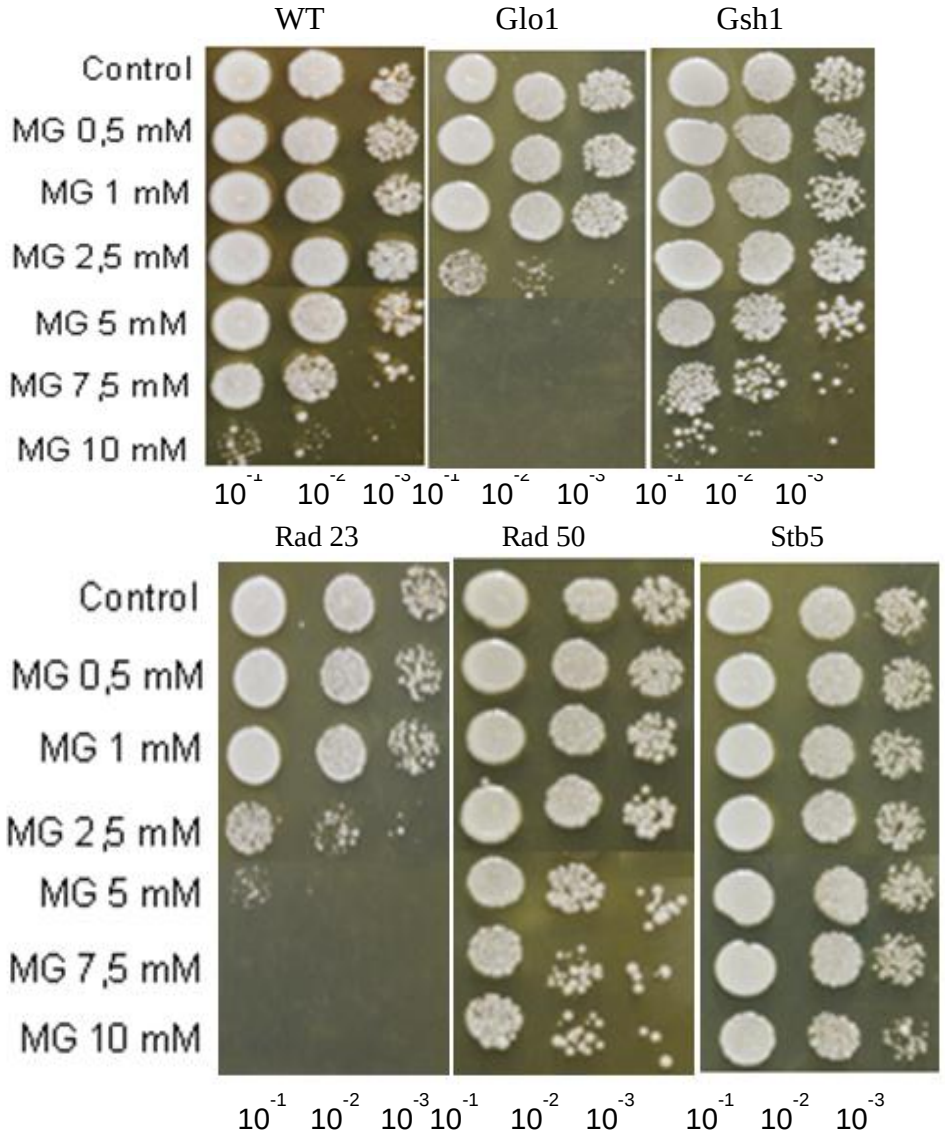


**Figure 4. Effect of MG on yeast mutant strains cell viability.** Yeast mutant strains were incubated in liquid YPDGal for 24 hours until saturation and then serially diluted ($10^{-1}$, $10^{-2}$, $10^{-3}$) in 300µL of sterile distilled water and spotted onto plates with YPDGal agar.

**Table 1.** List of the *S. cerevisiae* single-gene-deletion mutants

| | **Yeast protein name** | **Yeast function** |
|---|---|---|
| YAL015C | NTG1 | DNA binding |
| YLL015W | BPT1 | Bilirubin transmembrane transporter activity |
| YLR043C | TRX1 | Disulfide oxidoreductase activity |
| YLR109W | AHP1 | Antioxidant activity |
| YML007W | YAP1 | DNA binding |
| YML028W | TSA1 | Antioxidant activity |
| YML032C | RAD52 | DNA strand annealing activity |
| YML061C | PIF1 | ATP binding |
| YMR201C | RAD14 | DNA binding |
| YOR028C | CIN5 | DNA binding |
| YOR040W | GLO4 | Hydroxyacyl glutathionehydrolase activity |
| YOR368W | RAD17 | DNA binding |
| YOR380W | RDR1 | DNA binding |
| YOL043C | NTG2 | Catalytic activity |
| YOL049W | GSH2 | Glutathione synthase activity |
| YOL028C | YAP7 | Sequence-specific DNA binding |
| YPL188W | POS5 | ATP binding |
| YPL194W | DDC1 | DNA binding |
| YBR216C | YBP1 | Molecular function |
| YBR244W | GPX2 | Glutathione peroxidase activity |
| YDR076W | RAD55 | ATP binding |
| YDR098C | GRX3 | Disulfide oxidoreductase activity |
| YDR369C | XRS2 | Double-strand telomeric DNA binding |
| YDR419W | RAD30 | DNA binding |
| YEL017W | GTT3 | Molecular function |
| YEL037C | RAD23 | DNA damage repair |
| YER042W | MXR1 | Oxidoreductase activity |
| YGR209C | TRX2 | Disulfideoxido reductase activity |
| YHL009C | YAP3 | Sequence-specific DNA binding |
| YHR106W | TRR2 | Thioredoxin–disulfidereductase activity |
| YHR178W | STB5 | DNA binding |
| YHR183W | GND1 | NADP binding |
| YCL033C | MXR2 | Metal ion binding |
| YKL026C | GPX1 | Glutathione peroxidase activity |
| YKL086W | SRX1 | ATP binding |
| YKL113C | RAD27 | Catalytic activity |
| YGR088W | CTT1 | Catalase activity |
| YLR364W | GRX8 | Glutathione-disulfidereductase activity |
| YLR288C | MEC3 | DNA binding |
| YDR217C | RAD9 | Double-strand DNA binding |

| | | |
|---|---|---|
| YPL091W | GLR1 | NADP binding |
| YPL022W | RAD1 | DNA binding |
| YFR044C | DUG1 | Dipeptidase activity |
| YGR234W | YHB1 | Nitric oxide reductase activity |
| YBL064C | PRX1 | Antioxidant activity |
| YGL058W | RAD6 | ATP binding |
| YNL191W | DUG3 | Contributesto gama glutamyl transferase activity |
| YNL241C | ZWF1 | NADP binding |
| YDR256C | CTA1 | Catalase activity |
| YKR042W | UTH1 | Molecular function |
| YDR272W | GLO2 | Hydroxyacyl glutathione hydrolase activity |
| YIL008W | URM1 | Proteintag |
| YIL010W | DOT5 | Antioxidant activity |
| YKR066C | CCP1 | Cytochrome-c peroxidase activity |
| YKR076W | ECM4 | Glutathione transferase activity |
| YIR018W | YAP5 | Sequence-specific DNA binding |
| YER095W | RAD51 | DNA binding |
| YLR032W | RAD5 | ATP binding |
| YML095C | RAD10 | ATP binding |
| YMR106C | YKU80 | ATP binding |
| YNL250W | RAD50 | DNA damage repair |
| YHR008C | SOD2 | Superoxide dismutase activity |
| YDR004W | RAD57 | ATP binding |
| YJR104C | SOD1 | Antioxidant activity |
| YBR281C | DUG2 | Contributesto gama glutamyltransferase activity |
| YCR066W | RAD18 | DNA binding |
| YJR052W | RAD7 | Contributes to DNA dependent ATPase activity |
| YJR035W | RAD26 | ATP binding |
| YBR010W | HHT1 | DNA binding |
| YBR014C | GRX7 | 2iron, 2 sulfur cluster binding |
| YNL099C | OCA1 | Hydrolase activity |
| YCL035C | GRX1 | Disulfide oxidoreductase activity |
| YCR083W | TRX3 | Disulfide oxidoreductase activity |
| YKR106W | GEX2 | Solute: hydrogenantiporter activity |
| YBR114W | RAD16 | ATP binding |
| YJL101C | GSH1 | Glutamate-cysteine ligase activity |
| YDL010W | GRX6 | Glutathione–disulfide reductase activity |
| YDL059C | RAD59 | DNA strand annealing activity |
| YDR453C | TSA2 | Antioxidant activity |
| YDR513W | GRX2 | Disulfide oxidoreductase activity |
| YER162C | RAD4 | DNA binding |
| YER173W | RAD24 | ATP binding |
| YER174C | GRX4 | Disulfide oxido reductase activity |
| YGL163C | RAD54 | DNA binding |
| YGL166W | CUP2 | Sequence-specific DNA binding transcription factor |

| | | |
|---|---|---|
| | | activity |
| YML004C | Glo1 | Glutathione metabolic process; methylglyoxal catabolic process to D-lactate |
| YGR258C | RAD2 | ATP binding |
| YPL059W | GRX5 | Disulfide oxidoreductase activity |

http://www.yeastgenome.org/

Table 2. Growth $IC_{50}$ values for mutant and wild type strains exposed to MG

| WT | Rad 23 | Rad 50 | Stb5 | Gsh1 | Glo 1 |
|---|---|---|---|---|---|
| 3.273 ± 0,16 | 1.622 ± 0,20 | 1.849 ± 0,23 | 4.644 ± 0,32 | 1.282 ± 0,20 | 0.665 ± 0,10 |

Data are presented as mean ± SEM (n=3).

# 4. CONCLUSÕES

## 4.1 CONCLUSÕES ESPECÍFICAS

De acordo com os dados obtidos no presente trabalho podemos concluir que:

- A toxicidade do MG variou com o meio de cultura, uma vez que as leveduras selvagens crescidas em meio contendo galactose foram mais sensíveis ao composto quando comparadas com aquelas crescidas em meio contendo glicose.

- Através do "screening" realizado sugere-se que a citotoxicidade do MG em *S. cerevisiae* está principalmente relacionada com danos ao DNA. De fato, cepas mutantes para genes ligados ao reparo de DNA e processos relacionados como Rad 23 e Rad 50 tiveram seu crescimento e viabilidade celular afetados pelo MG.

- Cepas mutantes para genes ligados às defesas antioxidantes como as enzimas superóxido dismutase, catalase, glutationaperoxidase e tioredoxinaredutase não foram sensíveis ao MG.

- A enzima glioxilase I exerce um papel regulatório importante na detoxificação do MG em *S. cerevisiae*, visto que a deleção dos genes Glio1 e Gsh1 aumentou a sensibilidade das leveduras ao MG.

## 4.2 CONCLUSÃO

Esse trabalho foi de grande importância por ser o primeiro “screening” realizado para verificar genes alvos à toxicidade do MG em *S. cerevisiae*, e pode servir de base para investigações dos mecanismos envolvidos nos efeitos tóxicos do composto em nível celular. Além disso, estudos que avaliem a citotoxicidade do MG podem contribuir significativamente para o entendimento de algumas complicações diabéticas e para busca por terapias mais efetivas.

## 5. PERSPECTIVAS

Tendo em vista os resultados obtidos nesse trabalho, temos como perspectivas:

- Usar as cepas encontradas como alvo da exposição ao MG em estudos voltados para a expressão gênica e morte celular por apoptose.

- Padronizar técnicas para avaliar parâmetros de estresse oxidativo em leveduras selvagens: peroxidação lipídica, produção de ROS, atividade de enzimas antioxidantes.

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