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Estudo do papel da homeostase do cálcio na reparação de danos no DNA em diferentes linhagens de *Saccharomyces cerevisiae*

NADINE PAESE POLETTO

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Dissertação apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, visando a obtenção do grau de Mestre em Biotecnologia.

Orientador: Prof. Dr. Diego Bonatto

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DISSERTAÇÃO APROVADA EM 21 DE NOVEMBRO DE 2008

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Dedico este trabalho às pessoas mais importantes da minha vida: ao meu pai, minha mãe, minha irmã e meu noivo.

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ESTRUTURAÇÃO DA DISSERTAÇÃO

A presente tese encontra-se estruturada da seguinte forma: uma introdução geral, os objetivos (geral e específicos), os três capítulos principais escritos na forma de artigos científicos (conforme as normas das revistas para as quais foram submetidos), uma discussão separada em subitens, conclusões (gerais e específicas) e perspectivas.

A introdução aborda alguns conceitos importantes para o entendimento dos processos de homeostase de Ca^{+2}, seus mecanismos de manutenção da concentração intracelular adequada, sua influência em relação a doenças humanas e mecanismos de reparação de DNA.

O primeiro capítulo, intitulado "THE CELLULAR CALCIUM HOMEOSTASIS AND ITS ROLES ON DNA REPAIR AND APOPTOSIS" trata de uma revisão sobre os mecanismos de homeostase de Ca^{+2}, sua importância, relação com patologias humanas, com mecanismos de reparação de DNA e apoptose, a ser submetido para o periódico *Molecular Genetics and Metabolism.*

O segundo capítulo, intitulado "EVALUATION OF CYTOTOXIC AND CYTOSTATIC EFFECTS IN *Saccharomyces cerevisiae* BY POISSONER QUANTITATIVE DROP TEST", aceito para publicação no periódico *Basic & Clinical Pharmacology & Toxicology* trata do desenvolvimento e aperfeiçoamento de uma técnica denominada de PQDT (*Poissoner Quantitative Drop Test*). Esta técnica diferencia-se das demais, pois propicia principalmente uma exposição crônica do modelo em estudo, propiciando a visualização de resultados e comportamento celular não observado nos demais testes.

O terceiro capítulo intitulado "RELATIONSHIP BETWEEN CALCIUM HOMEOSTASIS AND DNA REPAIR MECHANISMS INDUCED BY 4-NQO IN S*accharomyces cerevisiae*" submetido ao periódico *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* trata do estudo da sensibilidade de linhagens

deficientes para os mecanismos de homeostase de Ca^{+2} e para o gene *RAD4*, frente aos danos causados por 4-NQO. Este estudo teve, como base, o uso de ferramentas de Biologia de Sistemas para avaliar a interpolação entre os sistemas de reparação de DNA como as proteínas que controlam a homeostase de Ca^{+2}. Avaliou-se também os efeitos citostáticos e atraso e/ou parada de ciclo celular nas linhagens testadas frente à exposição ao agente genotóxico.

Na seqüência apresenta-se uma discussão geral inter-relacionando os resultados descritos nos capítulos acima, as conclusões e as perspectivas geradas por esta dissertação de mestrado.

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LISTA DE ABREVIATURAS

4-NQO	4-Óxido de nitroquinoleína
5-FU	*5-Fluorouracil* (5-Fluorouracila)
6-4PP	*6-4 Photoproducts* (6-4 Fotoprodutos)
ALS	*Amyotrophic Lateral Sclerosis ou Lou Gehrig's Disease* (Esclerose Amiotrófica Lateral)
BER	*Base Excision Repair* (Reparação por Excisão de Bases)
CPD	*Cyclobutane Pyrimidine Dimers* (Dímeros de pirimidina)
CRP	*Serine Protease* (Serina protease)
DSB	*Double Strand Breaks* (Quebras duplas de fita de DNA)
EA_2	*Episodic Ataxia type 2* (Ataxia episódica do tipo 2)
EC_{50}	*Effective concentration giving 50% response* (Concentração efetiva com 50% de resposta)
EGF	*Epithelial Growth Factor* (Fator de crescimento epitelial)

ER	*Endoplasmic Reticulum* (Retículo Endoplasmático)
FK506	Tacrolimus
GC	*Golgi complex* (Complexo de Golgi)
GGR	*Global Genome Repair* (Reparação global do genoma)
GO	*Gene Ontology* (Ontologia Gênica)
HR	*Homologus Recombination* (Recombinação Homóloga)
IP_3	*Inositol Triphosphate* (Inositol trifosfato)
LOEC	*Lowest observed effect concentration* (Menor concentração Efetiva observada)
MMR	*Mismatch Repair* (Reparação de erros de emparelhamento de bases)
MMS	*Methyl methanesulfonate* (Metil-metanossulfonato)
NER	*Nucleotide Excision Repair* (Reparação por excisão de nucleotídeos

NHEJ	*Non Homologus End-Joining* (Recombinação não homóloga)
P/QCC	*P/Q-type Voltage-dependent calcium channels* (Canais de cálcio dependentes de voltagem do tipo P/Q)
PPPI	*Physical protein-protein interaction* (Interação física proteína-proteína)
PQDT	*Poissoner Quantitative Drop Test* (Teste de Poissoner em gotas quatitativo)
ROS	*Reactive Oxigen Species* (Espécies Reativas de Oxigênio)
SCA	*Spinocerebellar Ataxia* (Ataxia espino-cerebelar)
SCAN1	*Spinocerebelar Ataxia with axonal neuropathy* (Ataxia espino-cerebelar com neuropatia axonal)
SERCA	*Sarcoplasmic/endoplasmic reticulum calcium ATPase* (ATPase dependente de cálcio do sarcoplasma/retículo endoplasmático)
SSB	*Single Stand Breaks* (Quebras simples de fita de DNA)
SSBR	*Single-strand break repair* (reparação de quebras simples de DNA)

TCR	*Transcription-couple repair* (Reparação acoplada à transcrição)
TDP1	*Tyrosyl DNA phosphodiesterase 1*
TFN	*Tumoral Factor Necrosis* (Fator de Necrose Tumoral)
TLS	*Translesion DNA Synthesis* (Síntese Translesão do DNA)
UVC	Luz Ultravioleta do tipo C (comprimento de onda de 260 nm)
VDAC	*Voltage-dependent anion-selective channel* (Canais seletivos de ânions e dependentes de voltagem)
VDCC	*Voltage-dependent calcium channels* (Canais de cálcio dependentes de voltagem)

RESUMO

O Ca^{+2} tem sido relatado como um íon de extrema importância celular. Sua concentração intracelular alterada está relacionada com: (i) a ativação de proteínas importantes, dentre elas as de reparação e de apoptose, (ii) as doenças humanas de grande relevância sócio-econômica e (iii) o aumento dos danos celulares. Sabendo-se que a sua concentração intracelular é responsável pela manutenção da viabilidade celular e frente à importância já conhecida deste íon, esta dissertação teve por objetivo, primeiramente, fazer uma busca sobre os dados existentes na literatura para delinear quais são os possíveis mecanismos moleculares pelo qual o Ca^{+2} afeta os mecanismos de reparação e apoptose. Esta busca norteou posteriores testes e pesquisas que culminaram nos resultados obtidos e apresentados neste trabalho.

Para a realização de alguns testes foi desenvolvida uma nova metodologia, o ensaio *Poissoner Quantitative Drop Test* (PQDT), a qual permite a análise de sobrevivência de diferentes linhagens de *Saccharomyces cerevisiae* deficientes ou proficientes em determinadas proteínas, frente à exposição crônica a um agente genotóxico. Esta técnica também permite observar efeitos citostáticos e citotóxicos nas células testadas.

Além disso, realizou-se uma análise de Biologia de Sistemas das proteínas de *S. cerevisiae* responsáveis pela homeostase de Ca^{+2} e encontrou-se uma correlação direta destas com proteínas responsáveis pela reparação de DNA por meio da via de excisão de nucleotídeos ou NER. Uma vez obtidos estes dados, as linhagens de leveduras deficientes nas proteínas tanto de homeostase de Ca^{+2}, Pmr1p, Cod1p, Por1p, como para o gene *RAD4* foram construídas. Além dos simples mutantes, foram construídas linhagens duplos mutantes para ambas as vias. Assim, foram realizados ensaios de citotoxicidade, citostaticidade e de parada do ciclo celular frente à exposição ao agente genotóxico 4-NQO. Conforme os resultados

obtidos, a homeostase de Ca^{+2} exerce influência sobre os mecanismos de reparação de DNA, principalmente nas proteínas da via NER, como a Rad4p. As proteínas Pmr1p e Cod1p são as proteínas de homeostase de Ca^{+2} mais importantes na resposta ao dano celular. Estes resultados propõem que o estresse de retículo endoplasmático e a fosforilação de proteínas de parada de ciclo celular foram os principais processos metabólicos evidenciados nas linhagens de leveduras testadas após a exposição ao agente genotóxico 4-NQO.

Os resultados da análise de Biologia de Sistemas e análise ontológica dos genes mostram que existem muitas vias bioquímicas relacionadas com a homeostase de Ca^{+2}. Isso demonstra a importância destes achados e predispõem muitos outros testes para a elucidação completa destas influências.

Palavras-chaves: *Saccharomyces cerevisiae*, homeostase de cálcio, reparação de DNA, 4-NQO

ABSTRACT

Calcium (Ca^{2+}) has been reported as an important cellular ion. The changes in the intracellular Ca^{2+} concentrations are related to: (i) the activation of major proteins related to DNA repair and apoptosis; (ii) induction of human diseases; and (iii) increase in cellular damages. Considering that the Ca^{2+} intracellular concentration is also responsible for the maintenance of cellular viability, the aim of this study was to proceed with a literature data mining to outline the major Ca^{2+} molecular mechanisms that can affect the cellular activities. This data mining was necessary to define further biological assays described in this work.

In this sense, it was developed a new methodology called PDQT, which allows to evaluate the survival of different *Saccharomyces cerevisiae* strains deficient or proficient for determined proteins in a chronic exposure conditions in the presence of a genotoxic agent. This new method also allows to observe the citotoxic, citostatic, and cell cycle arrests effects induced by a genotoxic agent.

Moreover, it was performed a Systems Biology analyses of the major *S. cerevisiae* Ca^{2+} homeostasis-associated proteins and it was found a direct association with proteins of the nucleotide excision repair (NER) pathway. The data gathered by Systems Biology analyses were used to construct yeast strains single and double mutants deficient for Ca^{2+} homeostasis-associated proteins, like Pmr1p, Cod1p, Por1p, and for the NER-associated protein Rad4p. The yeast strains obtained were challenged against the genotoxic agent 4-NQO and citotoxic, citostatic, and cell cycle arrest assays were performed. Taking into account the results, it was observed that Ca^{2+} homeostasis mechanisms affect the DNA repair pathways, specially the NER proteins like Rad4p. In this sense, both Cod1p and Pmr1p are the most important Ca^{2+} homeostasis proteins for the damage cellular response. In addition, these results propose that

ER stress and the phosphorylation of cell cycle arrest-associated proteins can be the major metabolic processes after 4-NQO exposure.

The results obtained by System Biology and gene ontology analyses proved that many biochemical pathways are related to Ca^{2+} homeostasis, which demonstrate the importance of the results obtained in this work and lead to other studies.

Keywords: *Saccharomyces cerevisiae*, calcium homeostasis, DNA repair, 4-NQO.

INTRODUÇÃO GERAL

O íon cálcio (Ca^{+2}) desempenha um importante papel na transdução de sinais externos e internos por meio do uso de proteínas específicas presentes no ambiente citoplasmático das células eucarióticas. Essa sinalização regula inúmeras funções celulares como a secreção, a proliferação, a sobrevivência celular, algumas formas de morte celular programada e expressão gênica. Assim, o Ca^{+2} desempenha um papel importante na transdução de informações do citoplasma para o núcleo, onde este íon pode ativar diferentes modelos de transcrição gênica (Santella & Carafoli, 1997). Além disso, tem sido observado que as alterações na concentração de Ca^{+2} citoplasmático participam de uma variedade de processos biológicos nas células de leveduras, incluindo o controle do ciclo celular, resposta ao acasalamento, dentre outros. Neste sentido, vários estudos demonstraram uma grande similaridade na homeostase de Ca^{+2} entre leveduras e eucariotos superiores (Santella & Carafoli, 1997).

Em *Saccharomyces cerevisiae* as proteínas que regulam a homeostase de Ca^{+2} são bem conhecidas. As principais são: (i) H^{+}/Ca^{+2}, presente no vacúolo (Dunn *et al*., 1994), (ii) Pmc1p, também presente no vacúolo (Atenbi & Fink, 1992), (iii) Pmr1p, presente no complexo de golgi (Cunningham & Fink, 1994), (iv) (iv) Cod1p, presente no retículo endoplasmático, (v) Por1p presente na mitocôndria (Blachly-Dyson *et al*., 1997), (vi) as proteínas citoplasmáticas calmodulina (Cmd1) e calcineurina (Cna1p) (Stewart *et al*., 1982) e (vii) Cch1p, situada na membrana plasmática (Tom & Rao, 2004).

Baseado na similaridade biológica entre *S. cerevisiae* e eucariotos superiores, esta levedura se mostra um modelo ideal, tanto bioquímico quanto molecular, para a compreensão da homeostase de Ca^{+2} associados à manutenção da integridade genômica, pois combina a facilidade de cultivo em laboratório com o grande número de ferramentas moleculares

disponíveis para a geração e a análise de linhagens mutantes específicas para diferentes vias bioquímicas.

Já foi relatado que, em seres humanos, os desequilíbrios na concentração intracelular de Ca^{+2} estão relacionados com arritmias cardíacas, fibrilação ventricular (Lakatta & Guarnieri, 1993), encefalomiopatias (Moudy *et al.,* 1995), infarto do miocárdio, câncer, Alzheimer e outras desordens do sistema nervoso central (Trump & Berezeski., 1995) e há evidências de sua associação com os mecanismos de reparação de DNA.

Assim, considerando a diversidade e a importância que os mecanismos de reparação de DNA possuem para a manutenção da viabilidade do organismo, tem sido demonstrado que os íons metálicos, especialmente o Ca^{+2}, desempenham um papel fundamental na ativação e controle destes mecanismos.

OBJETIVOS

OBJETIVO GERAL

Avaliar a influência da homeostase do íon cálcio (Ca^{+2}) nos mecanismos de reparação de DNA frente à ação do agente genotóxico 4-NQO em diferentes linhagens da levedura *Saccharomyces cerevisiae.*

OBJETIVOS ESPECÍFICOS

1. Avaliar a interação proteína-proteína para as vias de homeostase de Ca^{+2} e reparação de DNA utilizando, para tanto, programas específicos de Biologias de Sistemas.

2. Construir linhagens de *S. cerevisiae* duplos mutantes para os mecanismos de manutenção da homeostase de Ca^{+2} e para reparação de DNA utilizando a técnica de disrupção simples.

3. Avaliar a sensibilidade (curvas de sobrevivência) das linhagens simples e duplos mutantes de *S. cerevisiae*, defectivas ou proficientes para a homeostase de Ca^{+2} e/ou combinadas com mutações para o gene *RAD4* frente à ação do agente genotóxico 4-NQO.

4. Avaliar efeito citostático nestas linhagens, por meio de medições de área de colônia.

5. Avaliar atraso e/ou parada de ciclo celular nos simples e duplos mutantes, pelo percentual de células nas fases G_1, S e G_2/M, por meio de microscopia óptica.

Capítulo I

THE CELLULAR CALCIUM HOMEOSTASIS AND ITS ROLES ON DNA REPAIR AND APOPTOSIS

A ser submetido para o periódico **Molecular Genetics and Metabolism.**

THE CELLULAR CALCIUM HOMEOSTASIS AND ITS ROLES ON DNA REPAIR AND APOPTOSIS

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Short title: DNA repair, apoptosis and calcium homeostasis.

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ABSTRACT

Calcium (Ca^{2+}) has an important physiological role in the maintenance of cells in different prokaryotic and eukaryotic organisms. In this sense, the intracellular Ca^{2+} concentration should be keep at constant levels independently of the extracellular environment, a condition that is achieved by many biochemical processes that compose the Ca^{2+} homeostasis. Disturbances in Ca^{2+} homeostasis originate degenerative diseases, many of them as a result of DNA damages introduced physiological and environmental factors that also affects the cellular integrity. Interestingly, the maintenance of genome and cellular integrity relies in proteins that participate in the DNA repair processes, being many of these proteins Ca^{2+}-dependent. Despite our current knowledge about Ca^{2+} homeostasis and DNA repair the interplay between both mechanisms are little understood. Thus, the purpose of this review is to give a broad view of what is known about the interactions between DNA repair and Ca^{2+} homeostasis mechanisms in two biological models, *Saccharomyces cerevisiae* and *Homo sapiens*. These data will allow getting a better picture about the essential role of Ca^{2+} homeostasis in the maintenance of genome integrity.

KEYWORDS: calcium homeostasis, DNA repair, apoptosis, voltage-dependent anion-selective channel, *COD1, PMR1.*

INTRODUCTION

Calcium (Ca^{2+}) is a major ion that regulates numerous cell functions in prokaryotic and eukaryotic organisms, including but not being restricted to motility, secretion, proliferation, cell survival, some forms of programmed cell death (e.g., apoptosis) and gene expression. In complex organisms like metazoans, Ca^{2+} also regulates muscle contraction, neurotransmission, hormone secretion, cell mitosis. In this sense, Ca^{2+} has a role as second messenger for hormones stimulation and the Ca^{2+} signal is mediate through a family of Ca^{2+}-binding protein [1].

Considering its broad physiological roles, the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) should be kept at constant levels independently of the environment, and impaired regulation of $[Ca^{2+}]i$ often results in altered cellular physiology. For example, an inability to elevate $[Ca^{2+}]i$ to the required level following an excitation in the cardiac muscle is a major pathogenic mechanism in heart failure [2]. In contrast, excessive elevation of resting $[Ca^{2+}]i$ is deleterious to almost all cell types, and can be associated with either necrotic or apoptotic cell death [3]. Abnormal handling of $[Ca^{2+}]i$ in the heart may induce severe arrhythmias and ventricular fibrillation [4]. Another relationship occurs with hemolytic diseases and Ca^{2+} homeostasis, where the external and intracellular gradient of Ca^{2+} can contribute to cell death [5].

It has been proposed that increased reactive species production by mitochondria is higher in the presence of exogenous Ca^{2+}; moreover elevate intracellular Ca^{2+} levels present in muscle and neural cell may exacerbate oxidative injury and contribute to the manifestation of mitochondrial encephalomyopathies [6]. In addition to mitochondria, endoplasmic reticulum (ER) is also one of the most important organelles related to Ca^{2+} homeostasis, being necessary to protein synthesis and folding. Alterations in Ca^{+2} homeostasis, by a variety of

environmental insults, as well as genetic diseases is associated with the accumulation of misfolded proteins, can affect the structure, function and integrity of the ER, resulting in ER stress [7]. Prolonged ER stress leads to organelle damage and dysfunction, and ultimately results in cell death [7]. Interestingly, neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, ALS (amyotrophic lateral sclerosis or Lou Gehrig's disease) and prion diseases are characterized by misfolded proteins and by its aggregation in insoluble bodies in cytoplasm and in different organelles, like ER [8, 9]. For example, the Alzheimer's β-amiloid aggregates accumulate in excessive amounts in the cell, overhelming the 'quality control' system designed to promote the correct folding and elimination of faulty proteins. In addition, β-amiloid peptides elevate rest levels of Ca^{2+} and enhanced Ca^{2+} responses to excitatory amino acids and ionophores in neurons, inducing neurotoxicity [8].

Defects in the repair of DNA damage have been associated with many human disorders such as cancer, immunodeficiency and neurodegeneration. Recent data suggest that defects in the repair or response to DNA SSBs (single strand breaks) may have particular impact on the nervous system and are associated with human ataxia [10]. It has been demonstrated that patients with Xeroderma Pigmentosum, a classical DNA repair disease, can develop progressive neurological abnormalities by defect repair of non-bulky DNA lesions [11].

In addition to the roles of Ca^{2+} on neurodegenerative diseases, the disturbed Ca^{2+} metabolism and a defective response to Ca^{2+} stimulations have been reported to exist in some of the patients with DNA repair deficiencies, showing the relationship with DNA repair and Ca^{2+} homeostasis [12]. A study investigating patients with ataxia-telangectasia, a DNA repair deficiency, indicated that the functional defect of T cell in ataxia-telangectasia is caused by defective Ca^{2+} dependent signal transduction in the CD3 complex [13].

DNA REPAIR

Many macromolecules that are necessary for cellular physiology are produced on basis of the genetic information stored in DNA. Cells genome is continuously threaded by reactive species like ROS generated by metabolism itself or by environmental agents like ultraviolet light, ionizing radiation. [14]. When replicating DNA polymerases are blocked by base lesions on the template strand, the replication forks may collapse, thereby resulting in double strand breaks (DSBs). In addition, depending on the type of lesions, certain DNA polymerases are capable of elongating DNA strands across damaged sites, and this translesion DNA synthesis (TLS) is frequently associated with replication errors, giving rise to mutations [15].

The DNA damage can be classified as mutagenic or genotoxic. From all types of lesions characterized so far, mutagenic changes are common in DNA and corresponds to small changes in the baseparing regions as spontaneous deamination, depurination oxidized bases, A:T to C:G and C:G to A:T transversions and A:G or T:C transitions. Failure to repair such DNA lesions is known to cause mutations that can lead to genomic instability, cell death or lifethreatening diseases like cancer in higher eukaryotes [16].

On the other hand, genotoxic lesions are a group of DNA chemical lesions like DSBs, UV-induced bulky adducts, DNA interstrand crosslinks, uncapped telomeres and certain oxidized bases. If these lesions are not repaired, the transcription, the DNA replication or chromosome segregation are blocked, leading to cell cycle arrested or apoptosis [14]. Thus, the maintenance of the DNA integrity is vital for the well functioning of every cell. Considering the diversity of DNA lesions found in this molecule, its long cellular half-life and the importance for information storage, it is not a surprise that there are a large number of proteins that have evolved to function in DNA repair and/or damage tolerance processes [17, 18].

The DNA repair is a complex process that uses several proteins with distinct functions, allowing the organism to overcome spontaneous mistakes in the DNA sequence as well as genotoxic lesions caused by physiological and environmental agents. The DNA repair processes can be grouped into three discrete mechanisms: (I) direct repair, (II) recombinational repair, and (III) excision repair [19]. There are tree types of excision repair that have been described: base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) [17]. BER involves primarily the repair of small, helix non-distorting base lesions and abasic sites. It is believed that repair of oxidative damage is mediated primarily by this pathway [20]. NER removes bulky adducts such as pyrimidine dimers resulting from exposure to various environmental agents. NER removes a broad variety of DNA lesions, including cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) generated by UV light, through two related subpathways. The general global genome repair (GGR) removes DNA damages from the entire genome, and the transcription-coupled repair (TCR) corrects lesions located on actively transcribed genes [21]. MMR corrects nucleotide mismatches that occur during replication or recombination [17].

Other types of DNA repair are homologous recombination (HR) and non homologous end-joining (NHEJ). HR is considered an error-free pathway since it uses a copy of the damaged segment as a template to guide repair. NHEJ is considered an error-prone pathway, since free ends are joined without the use of a template or through use of very small microhomologous repeats and, consequently, there often may be an associated loss of nucleotides or translocation [17]. Double-strand break repair (DSBR) uses NHEJ or HR repair pathways to repair DNADSBs [22]. These repair mechanisms can work alone or in group.

Additionally to DNA repair, cell cycle control, senescence and induced cell death by apoptosis comprising the so called cellular DNA damage response [14].

Defects in human DNA repair mechanisms can lead to several diseases like Fanconi's Anemia, Xeroderma Pigmentosum, Ataxia Telangectasia, Cocayne Syndrome and others malignancies. Some these diseases can lead to cancer development and neurodegeneration [17].

Many proteins that act in repair mechanism are calcium-dependent to own activities. Because of it, the aim of this study is to correlate responsible of Ca^{2+} homeostasis proteins with DNA repair proteins and apoptosis. After it to elucidate how occur this pathways interactions.

MAJOR EUKARYOTIC CALCIUM HOMEOSTATIC MECHANISMS

As indicated before, the $[Ca^{2+}]i$ control is made by organelles like ER and mitochondria. However, other organelles like golgi complex (GC), vacuole/lysosomes, and nucleus are also necessary to keeps the adequate $[Ca^{2+}]i$. Based on the Ca^{2+} uses by the cell as a signaling molecule, the Ca^{2+} gradients across the plasma membrane and Ca^{2+} stores in organelles must be maintained.

At the plasma membrane, high-affinity Ca^{2+} influx is mediated by a complex consisting of Cch1, a homolog of the mammalian voltage-gated α_1-subunit of Ca^{2+} channels, and Mid1 a putative stretch-activated Ca^{2+} channel [23].

In mammalian cells the RE mechanism of Ca^{2+} control is based on sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pumps [24 to 27]. There are more than one type of SERCA in mammalian cells, SERCA1 and SERCA2. The physiological role of SERCA1 of mediate Ca^{2+} uptake into skeletal muscle was recently demonstrated in Brody's disease in humans, which is characterized by impaired skeletal

muscle relaxing [28]. SERCA2 comprises the Ca^{2+} cardiac pumps and the major intracellular Ca^{2+} pump in the smooth muscle or non muscle tissue [29].

In the yeast *S. cerevisiae* the Ca^{2+} homeostasis proteins are better described and some of its biochemical functions are known. The major proteins are: (i) the vacuolar pump H^+/Ca^{2+}, (ii) the vacuolar Pmc1p, (iii) the GC Pmr1p, (iv) the ER Cod1p, (v) the mitochondrial Por1p, (vi) and the cytoplasmic calcineurin and calmodulin.

Biochemical studies indicate that the yeast vacuolar membranes transport Ca^{2+} by an H^+/Ca^{2+} pump. This Ca^{2+} uptake is completely dependent of transmembrane pH gradient that is produced by vacuolar H^+/V-ATPase [30].

The Pmr1p belongs to the ATPases family of sarco/endoplasmic reticulum. This protein is responsible for Ca^{2+} transport to GC to support many secretory functions [31]. Genetic evidences suggest that it acts as a transporter supplying Ca^{2+} to other compartments in the secretory pathway. The *pmr1*Δ mutants cells secrete abnormal proteins that are not cleaved by proteases Ca^{2+}-dependent locate in GC, a condition that can be reverted by Ca^{2+} supplementation [32]. The ability of *pmr1*Δ mutants to tolerate external Ca^{2+} suggests that additional transporters are also important to control citosolic Ca^{2+}. Greber and Gerace [33] found that the reduction of Ca^{2+} concentrations in ER lumen and in the nuclear membrane caused by ionophores and thapsigargin (an ER-pump Ca^{2+} inhibitor), inhibits irreversibly proteins signalling through the pore and also the transport of small molecules. In another study it was described that the protein transport trough the nuclear intraenvelop pores is mediated by calmodulin, in a GTP-dependent manner [34]. In this sense, the nuclear envelop is an extension of ER and can also be consider a Ca^{2+} deposit.

The steady-state 0.1 μmol L^{-1} intracellular concentration is maintained by means of the combined action of Pmr1p in the GC, Pmc1p in the vacuole, the H^+/Ca^{2+} antiporter and other possibly unidentified transporters [30]. Interestingly, mutant *pmc1*Δ grow poorly in media

containing high Ca^{2+} concentration, although growth can be restored by overexpression of *PMR1*. When FK506 (Tacrolimus) is added into the culture medium, it can restores the growth of *pmc1*Δ mutants in high Ca^{2+} concentration. Cyclosporin A, an inhibitor of calineurin, also restores growth of *pmc1*Δ mutants in elevated Ca^{2+} media [32]. Thus, the role of Pmc1p in Ca^{2+} tolerance may be shared by Pmr1p [31]. This possibility was verified considering that the overexpression of *PMR1* in *pmc1*Δ mutants can restore the growth of these yeast strains in high Ca^{2+} media. Moreover, simultaneous deletion of both *PMC1* and *PMR1* causes a phenotype more severe than either mutation alone. The double mutant strain *pmr1*Δ*pmc1*Δ is enviable in all media despite the levels of external Ca^{2+} external. However, this lethality can be prevented by inactivation of calcineurin with FK506 [31].

Cronin *et al*. [26] identify another yeast ATPase responsible for RE Ca^{2+} regulation, Cod1p. These researchers found a Ca^{2+} suppression phenotype, demonstrating that this protein could also be responsible to supply Ca^{2+} to the RE. Cod1p works with Pmr1p in glycoprotein processing, biosynthesis, and RE quality control [35]. Another study correlating Pmr1p and Cod1p verify that *pmr1*Δ mutants have 50% reduction of Ca^{2+} in RE [36], proving that Pmr1p is important to RE activity despite this protein is in another organelle [25]. It should be noted that the lumen of the ER is a unique environment, containing the highest concentration of Ca^{2+} within the cell because of active transport of Ca^{2+} ions by the ATPases. Release of Ca^{2+} from the ER plays a critical role in cellular signaling by the second messenger inositol triphosphate (IP3) and cytosolic ADP-ribose and other regulators via effects on the IP3 receptors. Various stimuli that cause the ER to dump Ca^{2+} precipitate cell death, including hypoxia, oxidants, and stimulators of IP3 production and pharmacological antagonists of SERCA [37].

Another organelle that is important to keep the Ca^{2+} homeostasis in the cell is mitochondria. Mitochondria take part in several cellular process like survival regulation, and

can block the induction of proteins associated to apoptosis by accumulation and release of Ca^{2+} [38]. In this sense, the transport of proteins and Ca^{2+} is mediate by the inner and outer mitochondrial membranes. The inner membrane have own transporters and permeases. Nevertheless, in the out membrane it believed that the transport occur by mammalian and yeast VDAC complex (voltage-dependent anion-selective channel) [38].VDAC is known to be the major complex that control the flux of compounds and ions to the outer membrane [39]. The VDAC Ca^{2+} permeability is regulated by citoplasmatic Ca^{2+}, which can affect many aspects of bioenergetic control [38].

The VDCA complex in yeast is codified by the *S. cerevisiae* genes *POR1* and *POR2* [39]. Yeast strains *por1*Δ have respiratory deficiencies, a phenomenon associate with a defective movement of metabolites through outer membrane [39]. By its turn, yeast double mutant strains *por1*Δ*por2*Δ can not grow in non fermentable carbon sources. However, these mutants grow, but many cells presents reduction in their metabolism. These mitochondria are capable to produce a membrane potential but can not convert this energy in ATP [39].

In addition to mitochondria, the cytoplasm is an important point to control the $[Ca^{2+}]i$. The cytoplasmic pool of Ca^{2+} is increased by external influx of Ca^{2+} and is decreased by organelle sequestration. Elevations of citosolic free Ca^{2+} concentrations, activates calcineurin by increasing the binding of Ca^{2+} and calmodulin [34].

The yeast phenotype of calcineurin mutants shows that calcineurin participates in regulating the intracellular concentration of several ions, because the mutants fail to grow when high concentrations of different ions (Na^{+}, Li^{+}, Mn^{2+}) are included in the growth medium [34].

Interestingly, there are models that shows that the action of Pmr1p and Pmc1p are essential for yeast growth and to prevent the inhibition of calcineurin by calmodulin in the presence of a high $[Ca^{2+}]i$ [34].

The phosphatase activity of calcineurin in mammalian cells cultures is stimulated by Ca^{2+} binding and calmodulin [40]. Ca^{2+}/calmodulin activate calcineurin *in vitro* by means of its interaction with the carboxy-terminal domain of catalytical subunit [41, 42].

In this sense, it is clear that Ca^{2+} play an important role in cells and probably the homeostasis of this ion can influence the DNA damage and/or repair mechanisms.

THE INFLUENCE OF CALCIUM HOMEOSTAIS ON DNA DAMAGE TOLERANCE AND/OR REPAIR

As described before, problems associated with the regulation of $[Ca^{2+}]i$ is related to a variety of acute and chronic diseases. These diseases include myocardial infarction, stroke, cancer, Alzheimer's disease, and others nervous system disorders [3]. Considering the importance of several DNA repair mechanisms that keep cell viability as well as the necessity to maintain a balanced concentration of Ca^{2+}, it is not a surprise that Ca^{2+} play a fundamental role in the activation and control of DNA damage tolerance and/or repair mechanisms. Gafter *et al.* [12] studied the activation of DNA repair mechanisms in human lymphocytes after sub-letal doses of UVC irradiation and showed that this activation depends on the $[Ca^{2+}]i$. In addition, it was verified that the DNA polymerase β, one of the principal polymerases of BER and NER depends of Ca^{2+} to act in DNA damage caused by ROS, UVC and ionizing radiation [43].

Also in mammalian cells, an elevated $[Ca^{2+}]i$ may initiate a cascade of signaling leading to activation of phospholipases A and C endonucleases, proteases, and the expression of several genes that may be important for the initiation and progression of cell death [3]. Interestingly, and despite our knowledge about the Ca^{2+} homeostatic mechanisms in mammalian and yeast cells, there is little data about the regulation of nuclear Ca^{2+}

concentrations. Studies using several fluorescent probes have suggested that after various stimuli or injuries the nuclear Ca^{2+} concentration may differ from cytoplasm, indicating that there is no free diffusion of Ca^{2+} through the nuclear pores. Gradients may also exist for Na^{+} and K^{+} and the nuclear envelope has been found to exhibit a high electrical resistance. The mechanism(s) that are involved in the maintenance of Ca^{2+} gradients between cytoplasm and nucleus are poorly understood; however, Nicotera *et al*. [44] reported that isolated nuclei accumulate Ca^{2+} in an ATP-dependent fashion. This data was also supported by Himpens *et al.* [45], which observed in a smooth muscle cell lineage that the normally higher cytoplasmic and nuclear gradients was reversed by ATP or epithelial growth factor (EGF) in a process that was modulated by tyrosine protein kinases, protein kinase C, and the Ser/Thr protein phosphatases 1 and 2A. The activation of nuclear kinases and phosphatases by Ca^{2+} could be related to the control of DNA damage tolerance and/or repair mechanisms. In this sense, it was reported for H_2O_2-induced cellular DNA damage a sustained rise in intracellular Ca^{2+} that, by its turn, triggers the cleavage of DNA by the activation of a Ca^{2+}-dependent endonuclease [46]. Moreover, mononuclear cells treated with genistein, a calcium chelator, showed 40% reduction in DNA repair after damage induced by H_2O_2, suggesting that Ca^{2+} signaling, via tyrosine kinase, was involved in oxidative DNA damage repair. Interestingly, it was reported in lymphocytes that the DNA repair mechanisms are activated after UV irradiation, and this induction is affected by cytosolic Ca^{2+} levels [47]. UV has also been shown to cause Ca^{2+} signaling by inducing tyrosine phosphorilation within seconds after irradiation [48]. In this sense, it was suggested that the Ca^{2+} signal generated during UV irradiation is essential for DNA repair, and UV-induced DNA synthesis, and cells treated with genistein have a low rate of DNA synthesis after UVC treatment [12].

Voltage-dependent calcium channels (VDCC) are hetero-multimeric complexes that mediate calcium influx into cells in response to changes in membrane potential. The α1A

subunit, encoded by the *CACNA1A* gene, is the pore-forming structure specific to the neuronal P/Q-type voltage-dependent calcium channels (P/QCC), present exclusively in neurons [49]. Mutations in this Ca^{2+}-channel are related with disease like Spinocerebellar Ataxia, Migraine and Episodic Ataxia Type 2.

On the other hand, recent data suggest that defects in the repair or response to DNA SSBs may have particular impact on the nervous system and are associated with human ataxia. Disorders in DNA repair typically cause additional symptoms such as mental retardation, photosensitivity, immunodeficiency and neoplasia [10]. In this sense, the autosomal dominant spinocerebellar ataxias (SCAs) are a complex group of neurodegenerative disorders characterized by progressive cerebellar ataxia of gait and limbs variably associated with ophthalmoplegia, pyramidal and extrapyramidal signs, dementia, pigmentary retinopathy and peripheral neuropathy [50]. Spinocerebellar ataxia with axonal neuropathy (SCAN1) is a human disease that is associated with mutation of TDP1 (tyrosyl DNA phosphodiesterase 1) protein and with a defect in repairing certain types of SSBs [10]. TDP1 is sequestered into multi-protein single-strand break repair (SSBR) complexes by direct interaction with DNA ligase III alpha and that these complexes are catalytically inactive in SCAN1 cells. These data implicate this process in the maintenance of genetic integrity in post-mitotic neurons [51].

Episodic ataxia type 2 (EA2) is caused by mutations within the α1A subunit of P/Q-type voltage-dependent calcium channel gene, *CACNA1A*. These channels are expressed throughout the brain and at neuromuscular junction, are implicated in the control of membrane excitatory and neurotransmitter release [52]. Most mutations responsible for EA2 are nonsense mutations that result in a truncated protein product and a nonfunctional channel. The EA2 mutation appears to result in a reduction of channel activity. This pathology is

common in childhood and adolescence and is characterized by episodes of nistagmus and ataxia associated with vertigo, diplopia, nausea, vomiting and headache [53].

Hemiplegic migraine is a unique migraine syndrome characterized by episodes of hemiplegia and hemianesthesia followed by headache. To date, mutations in 2 genes have been identified in families with hemiplegic migraine: *CACNA1A*, encoding neuronal calcium channel subunit, and ATP1A2, encoding a catalytic subunit of a sodiumpotassium–ATPase. Both of these genes code for transmembrane ion channels and transporters heavily expressed in the brain [54, 55].

In conclusion, this approach suggests the stimulus for the DNA repair synthesis was mediated by Ca^{2+} signaling through tyrosine protein kinase phosphorylation pathway [10].

CALCIUM AND APOPTOSIS

All stimuli that exceed the intensity of normal physiological response result in cellular injury and in the cellular reactions to injury that follows. Such stimuli include chemical, microbial, and physical agents, and deprivation of normal nutritional requirements, e.g., anoxia, ischemia, and other nutritional deficiencies. Following injury, cells undergo a series of reactions that constitute the basis of all disease [3]. These injuries induces DNA damages that need to be repair, or if the cell failed in these repair mechanism these damages can evolutes to cell death by apoptosis. Nucleases and proteases are important in apoptosis process. Apoptotic cells exhibit extra nuclear features and functional changes in the nucleus. Apoptosis frequently requires gene activation, may be regulate by nuclear proteins, and is characterized by the early condensation of chromatin, followed by DNA fragmentation [56]. Compelling evidences, indicates that the nuclear change typical of apoptosis can be initiating

by nuclear Ca^{2+} signal. The release of extracellular Ca^{2+} or its intracellular buffering components prevents the nuclear apoptotic changes [56].

Many studies provide evidences that increase in intracellular Ca^{2+} and also alterations in Ca^{2+} organelles homeostasis might be involved in triggering apoptosis [57]. It has also been reported by Bellomo et al. [58] that the exposure of adenocarcinoma cells to TNF-α induced an early intracellular Ca^{2+}, endonuclease activation and eventually apoptosis.

There are two basic theories to explain how Ca^{2+} homeostasis alterations can lead to apoptosis: (1) the Ca^{2+} intracellular elevation, by decrease of intracellular stores and influx across the plasma membrane, which can be caused by a signal transduction mechanism followed by the action of Ca^{2+}-activated proteases, Ca^{2+}-activates endonuclease, and transglutaminase activation or (2) by emptying of intracellular Ca^{2+} stores by Ca^{2+} chelators and catabolic enzymes [59].

Biochemically, apoptosis has been characterized by endogenous activation of specific endonucleases. The most interesting of the Ca^{2+}-dependent endonucleases is probably NUC18. This enzyme has a nuclear localization in numerous cell types and preexists in a repressed state in nonapoptotic cells [59]. It was first described by Gaido and Cidlowski [60] as a low-molecular weight nuclease with Ca^{2+} and Mg^{2+} depending activity in apoptotic lymphoid cells in response to several kinds of apoptotic stimuli. Many proteases play an important role in apoptosis like calpain [62]. An increase in calpain has been observed in apoptotic models preceding DNA fragmentation and morphological changes in cell [63]. Moreover, calpains cleave diverse proteins and are thought to participate in the induction of cellular necrosis in various cells types and the regulation of apoptosis. Another nuclear Ca^{2+}-dependent related protein and that is a substrate for calpain is calcineurin. Deregulation of these proteins has been implicated in the pathogenesis of several disorders, including hypertension, heart diseases, diabetes, cerebral ischemia, and Alzheimer's disease. The

calpain-mediated proteolytic cleavage of calcineurin increases phosphatases activity, which promote caspases-mediated neuronal cell death [64].

The prevention of rise intracellular Ca^{2+} concentration reduce the apoptotic induction, possibly because several nucleases and responsible factors of previous DNA degrade are Ca^{2+}-dependent [1].

Oxidative stress that is known to be commonly involved in apoptosis can disrupt Ca^{2+} homeostasis because Ca^{2+} transport system localized to the ER, mitochondria and plasma membrane can be damage by oxygen radicals [56].

In conclusion, it has been demonstrated that Ca^{+2} plays a role with development and regulation of many important enzymes and proteins responsible for DNA repair and apoptosis.

For this purpose, other studies are needed in order to describe the role of Ca^{2+} manly in stress response after injuries.

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Capítulo II

EVALUATION OF CYTOTOXIC AND CYTOSTATIC EFFECTS IN *Saccharomyces cerevisiae* BY POISSONER QUANTITATIVE DROP TEST

Artigo aceito para publicação

Basic & Clinical Pharmacology & Toxicology

Evaluation of cytotoxic and cytostatic effects in *Saccharomyces cerevisiae* by poissoner quantitative drop test

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Short title: Poissoner quantitative drop test

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Abstract

Biological models have long been used to establish the cytotoxicity and cytostaticity of natural and/or synthetic chemical compounds. Current assay techniques, however, typically require the use of expensive technological equipment or chemical reagents, or they lack adequate testing sensitivity. The poissoner quantitative drop test (PQDT) assay is a sensitive, inexpensive and accurate method for evaluation of cytotoxicity and/or cytostatic effects of multiple chemical compounds in a single experiment. In this study, the sensitivity of the PQDT assay was evaluated in a wild-type *Saccharomyces cerevisiae* strain using 4-nitroquinoline *N*-oxide (4-NQO) and methyl methanesulfonate (MMS), both cytotoxic and genotoxic standard compounds, and cytostatic 5-fluorouracil (5-FU), an antitumoral drug. Yeast cell colony growth was measured in culture media containing increasing concentrations of the two chemical agents. The results showed the PQDT assay was able to clearly differentiate the cytotoxic effect of 4-NQO and MMS from the cytostatic effect of 5-FU. Interestingly, the cytostatic effect of 5-FU followed an exponential decay curve with increasing concentrations, a phenomenon not previously described for this drug. The PQDT assay, in this sense, can be applied not only for cytotoxic/cytostatic assays, but also for pharmacodynamic studies using *S. cerevisiae* as a model.

Keywords: Poissoner device; *Saccharomyces cerevisiae*; cytotoxicity; cytostaticity; 5-fluorouracil; 4-nitroquinoline *N*-oxide; yeast colony area measurement.

1. Introduction

Cellular viability following DNA damage has been a topic of intense research in fields such as genotoxicity, toxicology, carcinogenesis, and cancer therapy [1]. Microorganism models can be used to evaluate cellular viability because they can be easily and inexpensively cultured in the laboratory. Additionally, their small size, simple morphology and large surface area in relation to their size gives microorganisms greater sensitivity than more complex organisms [2]. The yeast *Saccharomyces cerevisiae* is a model eukaryotic microorganism with a short generation time and very simple growth requirements. The molecular biology and genetics of this organism are well established. Mutants can be easily constructed and are commercially available, and plasmids and promoters for gene expression are well developed [3]. As such, *S. cerevisiae* has been used extensively as a model organism for the study of mammalian diseases and pathways and for evaluation of toxic compounds [4], such as genotoxic and cytotoxic agents.

Yeast cell colony growth can be monitored on agar plates containing dilutions of toxic agents to establish cytotoxicity/cytostatisticy of a compound [5]. Many toxic compounds introduce DNA damage and cause formation of bulky lesions that block DNA replication and/or RNA transcription, leading to cell cycle arrest and the inability of the cells to form colonies [6]. Current colony counting techniques and methods used to measure the size of yeast colonies have drawbacks that include: (i) inadequate sensitivity to determine the effect of low doses of cytotoxic/cytostatic compounds; (ii) high expense due to the need for specialized equipment or chemical substances [5]; and (iii) induction of starvation in treated yeast cells over time, an effect that has been shown to increase cellular resistance to chemical agents in mammalian cells [7]. For this study, we developed an improved assay method, the poissoner quantitative drop test (PQDT) based on the poissoner assay [8], to evaluate the sensitivity of yeast cells to genotoxic and cytotoxic compounds. Using the PQDT assay we

calculated (i) the number of colonies formed (cytotoxic analysis), and (ii) the sizes of colonies formed (cytostatic analysis) in a solid culture media containing increasing concentrations of tree chemical compounds. Therefore, we evaluated the toxicological effects of the common chemical agents, 4-nitroquinoline *N*-oxide (4-NQO; a genotoxic, cytostatic and cytotoxic standard agent) [9,10], methyl methanesulfonate (MMS; a genotoxic, cytostatic and cytotoxic standard agent) [11,12] and 5-fluorouracil (5-FU; an antitumoral, cytostatic agent) [13].

2. Material and methods

2.1. Yeast strain, culture media and growth conditions

The wild-type (WT) yeast strain BY4741 (*MAT*a *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) was purchased from EUROSCARF (Johan Wolfgang Goethe-University, Frankfurt, Germany). Complete glucose medium (YEPD) containing 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) glucose was used for routine yeast growth. Synthetic culture medium (SynCo) was used for PQDT assay and contained 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, and 2% (w/v) bacto-agar. The medium was supplemented with 1 mg histidine, 2 mg leucine, 2 mg methionine, and 2 mg uracil per 100 mL of SynCo. All yeast cultures were grown for three days at 28°C.

2.2. PQDT protocol, survival curve, yeast area colony estimation, and EC_{50}-LOEC determination

The poissoner assay uses a poissoner device (shown in Fig. 1), which is a circular brush-like stamp with 150-200 obtuse prongs made from stainless steel. The device fits inside a Petri dish. Each prong transfers small, equal-sized drops of the cell suspension onto the agar surface. The optimal diameter of the prongs (2.6 mm) and the optimal interval between each

prong (3.0 mm) ensures more uniform distribution than that observed with traditional plating techniques.

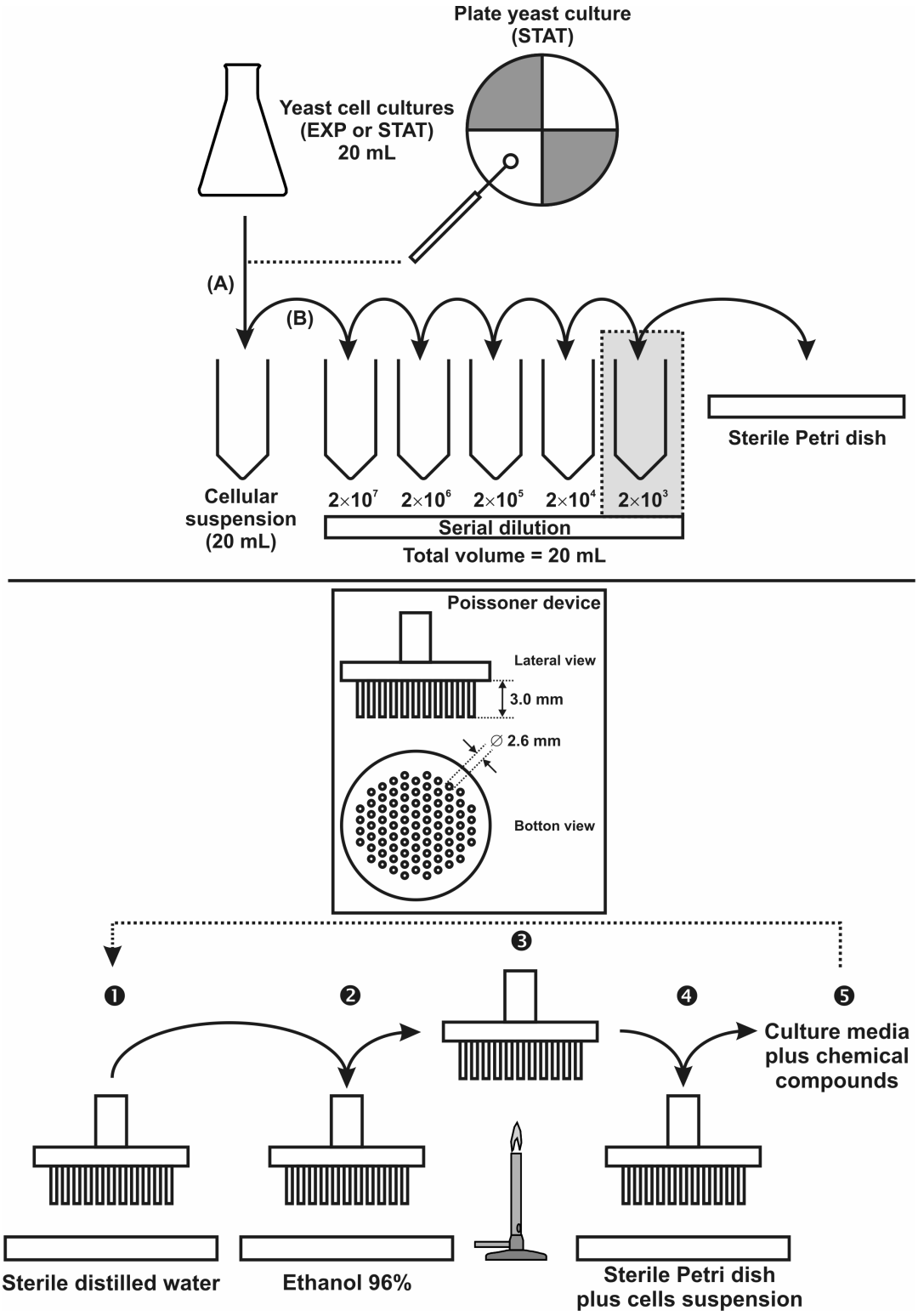

Fig. 1. Schematic drawing of the two steps of the poissoner quantitative drop test. In the first step a yeast cell suspension was prepared from an exponential (EXP) or stationary (STAT) culture made in liquid or

solid culture media, followed by the preparation of an initial cell suspension in saline solution (A; volume of 20 mL). The number of cells was estimated (B) and then the suspension was diluted to a final concentration of 2×10^7 cells/mL (volume of 20 mL). This suspension was serially diluted to a final concentration of 2×10^3 cells/mL (gray traced box) in 20 mL of saline solution and transferred to a sterile Petri dish. In step two, the poissoner was (1) washed with sterile double-distilled water, (2) immersed in ethanol 96%, (3) flamed, and (4) immersed in the cellular suspension prepared as described above. The drops were deposited on the surface of culture media containing different concentrations of chemical compounds. Once finished, the poissoner was prepared for another PQDT assay (dashed arrow). The central box contains a drawing of a poissoner device, shown from both a lateral and a bottom view. The length and diameters of pins are indicated in the figure.

The PQDT assay consisted of two steps: (i) yeast cell suspension preparation; and (ii) plating using the poissoner device (Fig. 1). Cell suspensions were prepared in 20 mL of sterile double-distilled water (ddH_2O) or saline solution (0.9% NaCl) from an exponential (EXP) or stationary (STAT) culture in YEPD medium. The number of cells/mL of the initial suspension was estimated by cell counting on a Neubauer chamber, and then an aliquot of suspension was withdrawn and used to make a second cell suspension containing 2×10^7 cells/mL (Fig. 1). This second cell suspension was serially diluted to a final cellular concentration of 2×10^3 cells/mL in 20 mL of sterile ddH_2O or saline solution.

The prepared suspension was transferred to a sterile Petri dish using the poissoner device. The device was first cleaned with sterile ddH_2O, then immersed in ethanol 96% (v/v), flamed, and finally immersed in cell suspension prepared as described above (Fig. 1). The poissoner device, containing the cell suspension, was gently pressed against a solid culture media, leaving small, visually distinct droplets on the agar surface. The poissoner device delivered about 0.2 mL of cell suspension, resulting in approximately 400 colonies per plate. After three days of growth at 28°C, the number of colonies was counted and the colony areas (in mm^2) were estimated from scanned images of Petri plates (gray scale; 150 dpi) using ImageJ analysis software (version 1.39 [http://rsb.info.nih.gov/ij/]).

In order to determine the EC_{50} (effective concentration giving 50% response) and LOEC (lowest observed effect concentration) values, a parametric curve was fitted to the data describing the survival curves of yeast cells treated with 5-FU, 4-NQO, and MMS. From this curve, the predicted compound concentration corresponding to 50% cell survival was calculated to be the EC_{50}. From the data obtained during the cytotoxicity assessment stage, a LOEC was also obtained, equal to the concentration at which a toxic effect produces 10% less cell proliferation than observed in the control.

3. Results and discussion

The poissoner assay was originally developed by Khromov-Borisov *et al*. [8] to control what is called the "extra-Poisson variability" or "overdispersion" of microbial cells on the gelified surface of culture media. This phenomenum occurrs with traditional plating methods and can lead to elevated variability of colony numbers in each experiment performed [8]. Because of the high variability, data from traditional assays are typically not reproducible. Until now, the poissoner assay has been used only to analyze the cytotoxic effects of chemical compounds, but improvements to the technique incorporated into this study made it possible to also evaluate cytostaticity induced by natural and/or synthetic compounds.

Using the improved PQDT assay, the effects of different concentrations of 5-FU, 4-NQO, and MMS on the survival (Fig. 2) and colony area (Fig. 3A to 3C) of the yeast wild-type (WT) strain BY4741 were determined. Cells plated in SynCo medium containing 5-FU (50 μM to 200 μM) went from nearly 100% survival at the lowest doses to 38% at the highest dose (Fig. 2). In contrast, yeast cells plated on Synco medium with 4-NQO from 0.1 μM to 0.5 μM showed a survival of 15% at the highest dose, with a linear dose-response for all 4-NQO concentrations tested (Fig. 2). Interestingly, a similar yeast survival curve was observed

when the cells were treated with different concentrations of MMS, with the cells having a survival of 29% at the highest dose (Fig. 2).

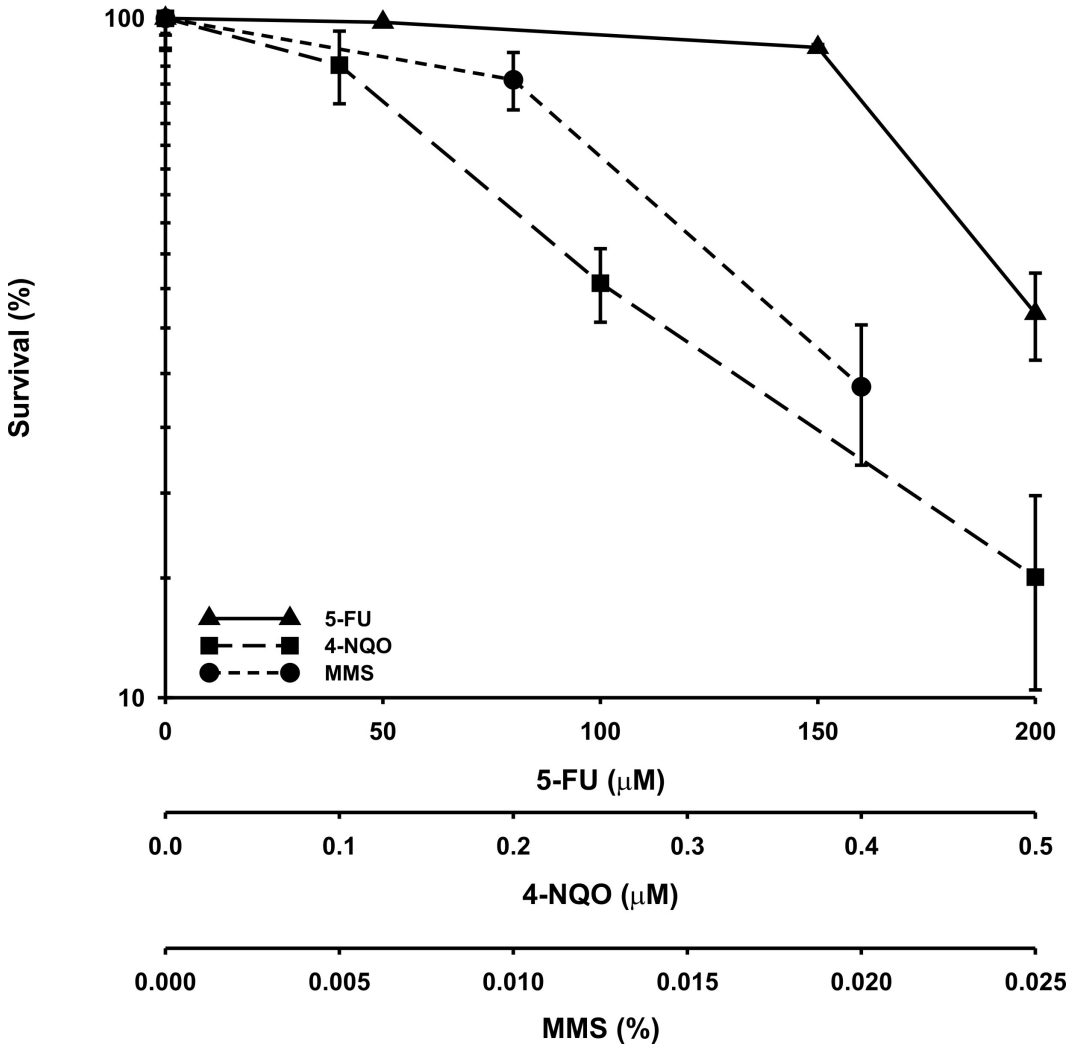

Fig. 2. Yeast survival profiles of BY4741 strain (WT) in 5-FU (solid line) and 4-NQO (dashed line) obtained from PQDT assay. The plot shows cell growth measured on synthetic culture medium containing different concentrations of 5-FU, MMS and 4-NQO (mean and standard errors of three independent assays).

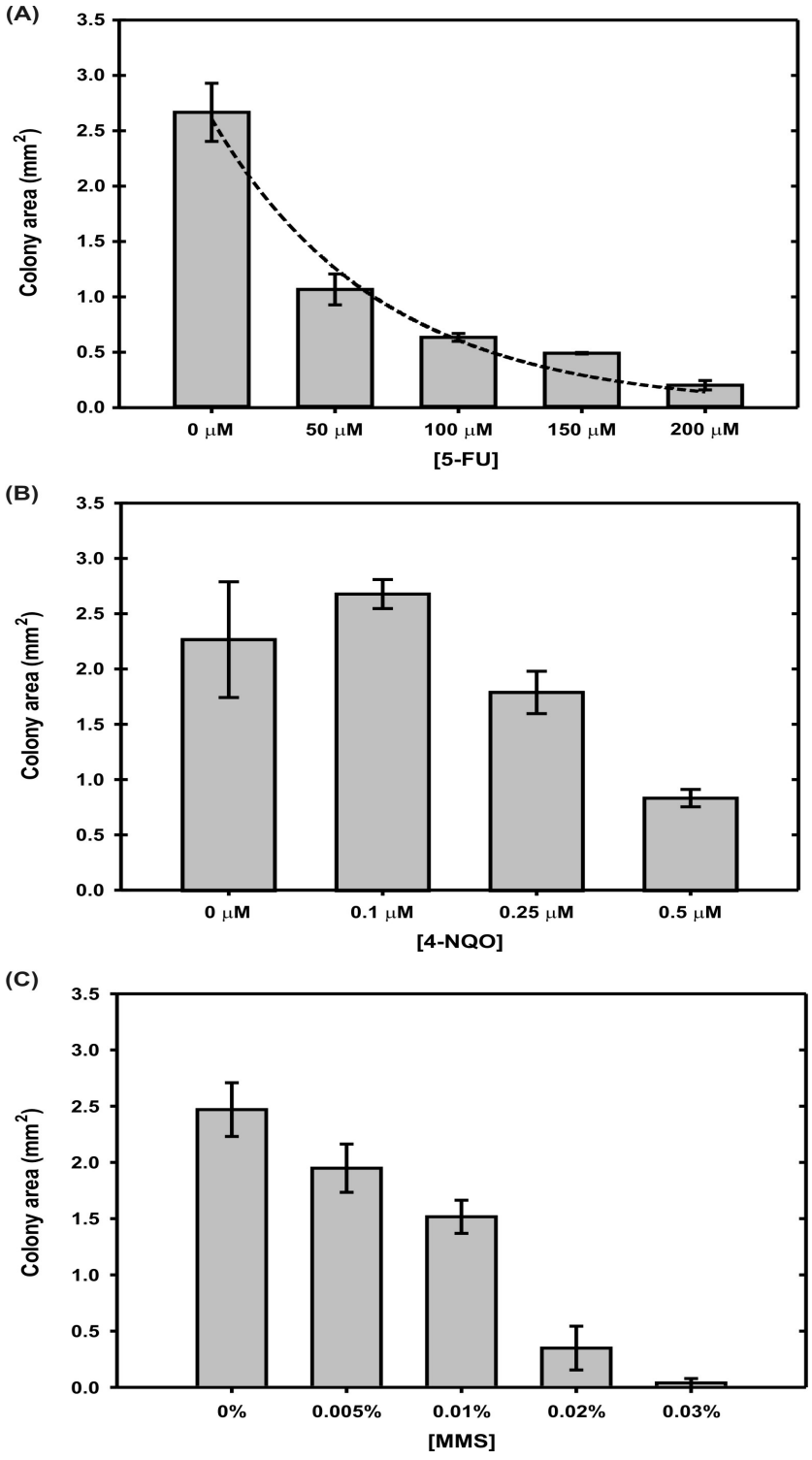

Fig. 3. Yeast colony area measurement (in mm^2) of BY4741 strain grown in the presence of 5-FU (A), 4-NQO (B) and MMS (C) after applying the PQDT assay. The dashed line in (A) represents exponential curve decay calculated from the colony areas of BY4741 strain exposed to different 5-FU concentrations. Error bars indicate the standard deviation of three independent experiments.

Cytostatic effects as measured by colony area occurred with both 5-FU (Fig. 3A), 4-NQO (Fig. 3B), and MMS (Fig. 3C) exposures; however the intensities of the effects were different. With both 5-FU and MMS, yeast cell colony areas decreased as 5-FU and MMS concentrations increased (Fig. 3A and 3C), with pronounced cytostaticity observed at 200 μM of 5-FU (Fig. 3A) and 0.03% of MMS (Fig. 3C). The cytostatic effect of 5-FU in mammalian cells is well described [13], and yeast cells are currently being used to study its mechanisms of action in the cell cycle and metabolism [14,15]. However, the exponential decay curve reported here for 5-FU in yeast is new and has not been shown for other antitumoral drugs. In contrast to 5-FU, cytostatic effects were only observed in yeast cells exposed to high doses of 4-NQO (Fig. 3B). The cytotoxic and genotoxic actions of 4-NQO and MMS are well documented, which makes both compounds useful as standards for mutagenic and genotoxic assays [9-12]. However, little is known about its cytostatic action in eukaryotic cells [9].

Considering the citotoxicity profile obtained for each compound test, it was possible to calculate the EC_{50} and LOEC values (Fig. 4A to 4C). It should be noted that 5-FU has high values of EC_{50} and LOEC (Fig. 4A) when compared to 4-NQO (Fig. 4B) and MMS (Fig. 4C), indicating that the major mechanism of 5-FU-induced cell growth inhibition is by cytostaticity instead of citotoxicity.

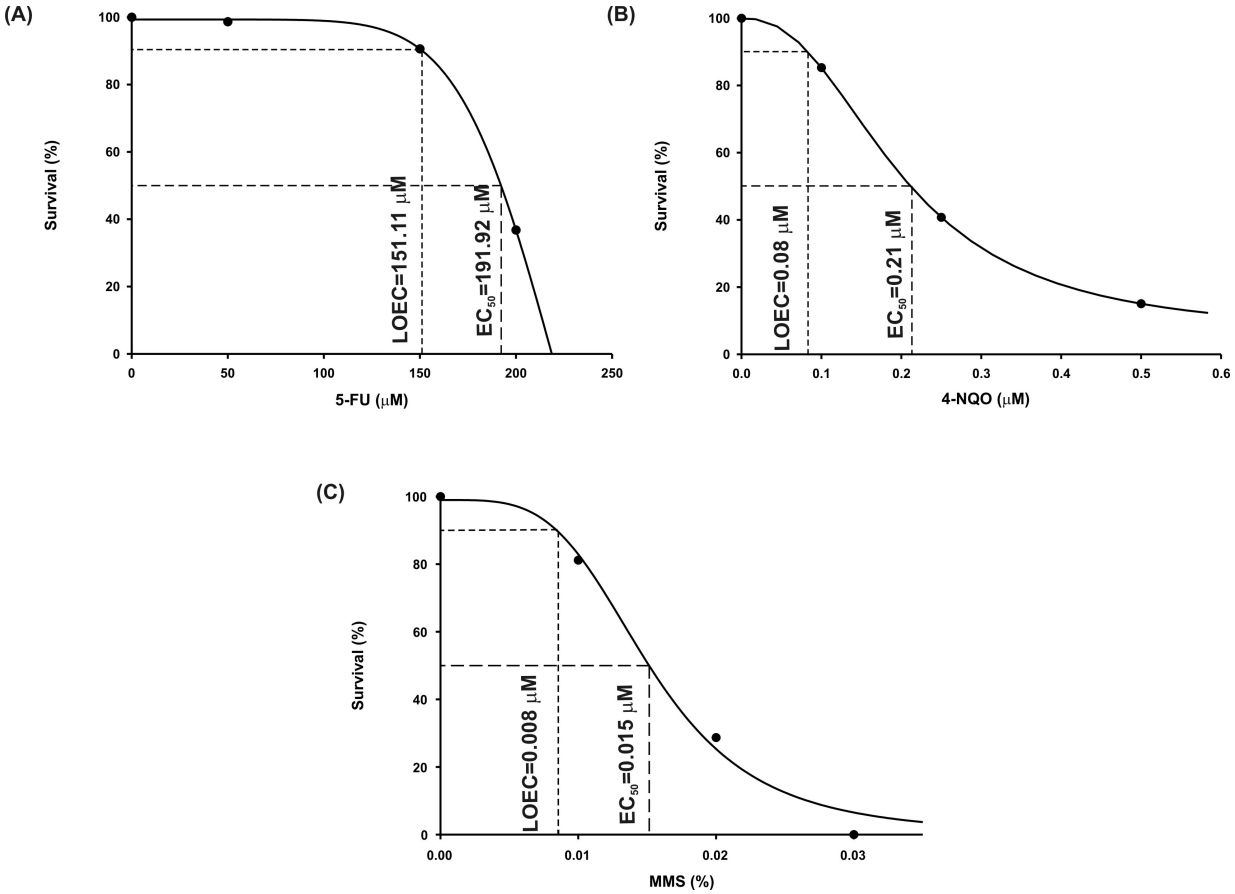

Fig. 4. Cytotoxicity profile for 5-FU (A), 4-NQO (B), and MMS (C) obtained from the yeast survival data. The EC_{50} and LOEC values are indicated in each graphic.

Yeasts have many advantages as a model organism when compared to *in vitro* cytotoxic assays using mammalian cells (e.g., fast cell cycle, prodigious growth, easily constructed mutants cells). In addition, the apparatus employed to culture mammalian cells are not easily available for many researchers. We found the PQDT method can be easily adapted for pharmacodynamic studies in yeast. It can be used to describe how compounds act in cells and to elucidate the mechanisms of action, a fundamental step for improving the efficacy of current antitumoral drugs. An additional advantage is the ability of the yeast cells to grow in the presence of chemicals that are directly incorporated in the culture medium, thus avoiding the induced effects of starvation on cellular resistance.

The PQDT assay is a new technique based on established yeast methodologies that can be used for simultaneously studying the cytotoxicity and cytostaticity of chemical compounds. Moreover, the technique can be implemented in any laboratory that works with microorganisms, a clear advantage when compared to other more expensive, equipment-intensive assays. The method has superior sensitivity over current cytotoxic and/or cytostatic analyses and gives reproducible results. We are currently conducting studies to expand the applicability of our PQDT assay and identify where the method is proficient and where it is deficient for different physiological mechanisms. Possibly, the new method can be used to discover new protein functions and/or describe the action of antitumoral drugs in the cell cycle, DNA repair, and longevity in yeast cells.

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Capítulo III

RELATIONSHIP BETWEEN CALCIUM HOMEOSTASIS AND DNA REPAIR MECHANISMS INDUCED BY 4-NQO IN *Saccharomyces cerevisiae*

Submetido para o periódico **Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis**

Relationship between calcium homeostasis and DNA repair mechanisms induced by 4-NQO in S*accharomyces cerevisiae*

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ABSTRACT

Calcium (Ca^{2+}) is a major ion that participates in cellular functions in both prokaryotic and eukaryotic organisms. In this sense, the maintenance of Ca^{2+} homeostasis by a small number of proteins is essential for the correct activity of these functions, especially DNA repair. Some evidences indicated that the increase in the intracellular Ca^{+2} levels are necessary for the activation of different DNA repair mechanisms. However, there is not a clear picture about what DNA repair and Ca^{2+} homeostasis mechanisms participate together in order to promote the repair and/or tolerance of genotoxic lesions. Thus, the aim of this study was to analyze what Ca^{2+} homeostasis mechanisms can influence DNA repair after damage induced by 4-NQO in *Saccharomyces cerevisiae*. For this purpose, we applied Systems Biology tools in order to verify the relationship between the proteins associated with Ca^{2+} homeostasis and DNA repair proteins. The data generated by Systems Biology was used to construct single and double yeast mutants cells for both mechanisms followed by cytotoxic and cytostatic analysis and cell cycle arrest after damage induced by 4-NQO. The results shown that the yeast mutants cells defective for Ca^{2+} homeostatic control present in the Golgi complex and endoplasmic reticulum (ER) are much more sensitive to 4-NQO than wild-type cell. Moreover, when these mutations where combined with the deletion of *RAD4* gene, which codifies for a nucleotide-excision repair protein detected by Systems Biology analyses, the cells showed a cell cycle arrest in G2/M phase after 4-NQO treatment. Taking together, the results indicate that alterations in the Ca^{2+} homeostatic mechanisms of Golgi complex and ER affect the repair of DNA damages induced by 4-NQO and these appears to be associated with ER stress and defective DNA repair by NER pathway.

KEYWORDS: calcium homeostasis, DNA repair, *Saccharomyces cerevisiae*, 4-NQO

1. Introduction

Calcium (Ca^{2+}) is the major ion that regulates many cells' functions in prokaryotic and eukaryotic organisms. Among their functions are secretion, motility, proliferation, cell survival, some forms of programmed cell death (e.g., apoptosis), gene expression, muscle contraction, neurotransmission, hormone secretion, and mitosis [1]. Considering its broad physiological roles the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) should be kept at constant levels independently of the environment, and impaired regulation of $[Ca^{2+}]i$ often results in altered cellular physiology. For example, the inability to elevate $[Ca^{2+}]i$ to the required level following an excitation is a major pathogenic mechanism in heart failure [2] and abnormal handling of $[Ca^{2+}]i$ in the heart may induce severe arrhythmias and ventricular fibrillation [3]. In contrast, excessive elevation of resting $[Ca^{2+}]i$ is deleterious to almost all cell types, and can be associated with either necrotic or apoptotic cell death [4]. Another relationship occurs with hemolytic diseases and Ca^{2+} homeostasis, where the external and intracellular gradient of calcium can contribute to cell death [5].

In S*accharomyces cerevisiae* this homeostasis is made by cellular organelles pumps and citoplasmic membrane. Among these proteins are: (i) Pmc1p, found in vacuole membrane [6], (ii) Pmr1p, situate in Golgi Complex membrane [7], (iii) Cod1p, present in the endoplasmic reticulum membrane [8], (iv) Cch1p, situate in plasmatic membrane [9], (v) Por1p situate in the outer mitochondria membrane [10], and Cmd1p and Cna1p, which are the citoplasmic proteins calmodulin and calcineurin, respectively [7].

Gafter *et al*. [11] studied the activation the DNA repair mechanism in human lymphocytes after sub-letal UVC irradiation and showed that an increase in $[Ca^{+2}]i$ is necessary for the activation of different DNA repair mechanisms. Moreover, it was also

verified that DNA polymerase β, one of the most important base excision repair (BER)- and nucleotide excision repair (NER)-associated polymerase have its function controlled by $[Ca^{2+}]i$ [12].

Considering the importance of several DNA repair mechanisms that keep cell viability as well as the necessity to maintain a balanced concentration of Ca^{2+} by a functional Ca^{2+} transport into the cells and organelles, it is not a surprise that Ca^{2+} play a fundamental role in the activation and control of DNA damage tolerance and/or repair mechanisms. However, it is not clear from literature what are the major Ca^{2+} homeostatic proteins that could be associated or participate with DNA repair mechanisms after a chemical insult. We conducted in a first moment a systems biology study to elucidate the significance of the relationship between Ca^{2+} homeostasis mechanisms and different DNA repair proteins from proteomic data available for the yeast *Saccharomyces cerevisiae*. The data generated by the systems biology analyses were further applied for the phenotypic study of specific single and double yeast mutant strains, defectives in Ca^{2+} homeostasis and/or DNA repair mechanisms after 4-NQO treatment.

2. Material and Methods

2.1 Binary protein-protein network design and topological analysis

A data mining screening and network design of binary physical protein-protein interactions (PPPI network) between Ca^{2+} homeostasis, DNA repair mechanisms and other biochemical process was performed with Cytoscape version 2.5.1 (http://www.cytoscape.org). For this purpose, a network containing 4,399 nodes and 34,630

edges describing the physical interaction among yeast proteins was downloaded from the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org). In addition, cPath, a Cytoscape plugin available as freeware at http://cbio.mskcc.org/cpath was employed to retrieve PPPI data from the cPath database.

The binary networks obtained from this first screening were analysed with Molecular Complex Detection (MCODE) software [13], with a Cytoscape plugin available at http://www.cytoscape.org/plugins2.php.The networks generated by MCODE were then combined in a unique binary PPPI network, also using the Cytoscape core plugin Merge Networks.

2.2 Gene ontology analysis

A gene ontology (GO) clustering analysis was performed with all proteins described in the PPPI network. For this purpose, the last version of Biological Network Gene Ontology (BiNGO) [14] and GOlorize tools [15] were used for the statistical evaluation of groups of proteins with respect to the present annotations of the Gene Ontology Consortium (http://www.geneontology.org). The degree of functional enrichment for a given cluster and category was quantitatively assessed (*p*-value) by hypergeometric distribution [15] and a multiple test correction was applied using the false discovery rate (FDR) [16] algorithm, fully implemented in BiNGO software. Overrepresented biological processes categories were generated after FDR correction with a significance level of 0.05.

2.3 4-NQO solution, yeast strains and plasmid, culture media and growth conditions

A stock solution of 4-NQO (0.025 μM and 0.0025 μM) was prepared in ethanol and stored at -20 ºC until use. The rich YPD medium, used for routine cell growth and survival analysis of *S. cerevisiae* strains (Table 1) contain 1% (w/v) yeast extract, 2% (w/v) bacto-petone, 2% (w/v) glucose, and 2% (w/v) bacto-agar. The synthetic complete medium (SC), used for cell cycle arrest and cells' survival after 4-NQO treatment contained 0.67% (w/v) yeast nitrogen base without amino acids and ammoniun sulphate, 2% (w/v) glucose, and 5% ammoniun sulphate supplemented with 2 mg adenine, 2 mg arginine, 5 mg lysine, 1 mg histidine, 2 mg leucine, 2 mg methionine, 2 mg uracil, 2 mg tryptophan, and 24 mg threonine per 100 mL SC. The omission SC media lacking uracil (SC-ura) was used to select yeast double mutant strains. The phenotype analyze was used SC media supplemented with 200 mM e 400 mM of a $CaCl_2$ solution. A saline solution [0.9% (w/v) NaCl] was employed for dissolution of cells suspension.

2.4 Construction of single and double mutant *S. cerevisiae* strains for DNA repair and/or Ca^{2+} homeostasis mechanisms

The *S. cerevisiae* mutant strains lacking the *RAD4* gene and Ca^{2+} homeostasis-associated genes was generated accordingly Guldner *et al*. (1996) [17]. Briefly, the plasmid (Table 1) was added to a polymerase reaction chain (PCR) with different oligonucleotides (Table 2) to generate disruptions cassettes. Each oligonucleotide (Table 2) contains 40 bp that correspond to the 5' and 3' regions of the gene that will be disrupted followed by 20 bp that correspond to the 5' and 3' regions of *URA3* gene present in pUG72. After PCR

generation of disruption cassettes, the *S. cerevisiae* single mutant strains were transformed by lithium acetate/PEG/ssDNA method as described by Gietz & Woods (2006) [18]. The potential double mutant yeast strains were selected in SC-URA. The characteristic Ca^{2+}-sensitivity phenotypes of three or four random colonies were scored in SC medium containing 200 and 400 mM $CaCl_2$.

Table1. *S. cerevisiae* strains and plasmid used in this study.

Strains/plasmid	Relevant genotype	Reference
BY4741	Mat **a;** *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0	Euroscarf
*pmr1*Δ	BY4741, except *pmr1::kanMX4*	Euroscarf
*rad4*Δ	BY4741, except *rad4::kanMX4*	Euroscarf
*por1*Δ	BY4741, except *por1::kanMX4*	Euroscarf
*cod1*Δ	BY4741, except *cod1::URA3*	This work
*pmr1*Δ*por1*Δ	*por1*Δ, except *pmr1::URA3*	This work
*pmr1*Δ*rad4*Δ	*rad41*Δ, except *pmr1::URA3*	This work
*cod1*Δ*por1*Δ	*por1*Δ, except *cod1::URA3*	This work
*cod1*Δ*pmr1*Δ	*pmr1*Δ, except *cod1::URA3*	This work
*cod1*Δ*rad4*Δ	*rad4*Δ, except *cod1::URA3*	This work
pUG72	*E. coli* plasmid; gene *URA3* with *loxP* sites. Resistance mark *ampR*.	Güldener *et al*. (1996)

Table 2. Oligonucleotides used to build single and double *S. cerevisiae* mutants.

Oligonucleotides	Base sequences
COD1A	5' – **AAA AAG GGG TAC TAC ATA AAA GAT TTA TCA TTT AGA GGC A**GC ATA GGC CAC TAG TGG ATC TG – 3'
COD1F	5' – **GAC CTA CAG AAA CAT AGG AAT CGG TAA ATG ACA AAG AAA TCA** GTC GAA GCT TCG TAC GC - 3'
PMR1R	5' – **ATG AAA GAT AAA GGG AAG GAA GGA AAA ACG ATA AAA GCA C**GC ATA GGC CAC TAG TGG ATC TG – 3'
PMR1F	5'- **GAA GCA AGG CCA GCA CAG ACG TAA GCT TAA GTG TAA GTA A**CA GTC GAA GCT TCG TAC GC – 3'

The distinction base sequence correspond to 5'-3' gene homology interest region

2.5 Survival and citostaticity assays

The yeast survival curves and the citostaticity showed by yeast strains to different concentration of 4-NQO were determinate accordingly to the Poissoner Quantitative Drop Test (PQDT) [19]. Briefly, a cell suspension containing 2×10^7 stationary cells ml^{-1} was prepared in saline followed by its serial dilution to 2×10^3 cells ml^{-1} in saline solution. This suspension was plated in solid YPD and solid SC previously prepared with different 4-NQO concentrations (0 nM, 0.5 nM, 1 nM, 2.5 nM, 10 nM, 0,05 μM, 0,1 μM, 0,25 μM, and 0,5 μM). The plates were incubated by 48 h at 28 ºC. Assays were repeated at last tree times and plating was made in triplicate for each dose. The sensitivity of yeast strains to 4-NQO was evaluated by counting the colonies forming unity (CFU).

The citostaticity of 4-NQO treatment in different yeast strains was determined by the measurement of colonies (in mm^2) obtained from the survival assay using the scanning images of Petri plates (150 dpi) followed by its analysis with ImageJ software, version 1.38 (http://rsb.info.nih.gov/ij). The graphs and statistical analysis (Two Way ANOVA, Levene, Kolmogorov-Smirnov) were made using Prisma version 4.0 (http://www.graphpad.com/prism) and SPSS for Windows version 16.0.

2.7 Analysis of cell cycle arrest

The cell cycle arrest after 4-NQO treatment was determinate by conventional optical microscopy by cell counting in Neubawer chamber followed by analysis of cell percentage arrested in G1, S and G2/M phase, accordingly Lewis *et. al.* (1999) [20]. Briefly, 5×10^6 cells ml^{-1} in STAT phase were incubate in liquid SC with different concentrations of 4-NQO (0,25 μM and 10nM), for 6 days at 28ºC. The counting was made every 48 h in triplicate.

3. Results

3.1 A protein-protein network of enzymes from Ca^{2+} homeostasis and DNA repair mechanisms in yeast

The large number of biological data available from high throughput experiments was used to identify thousands of pairwise PPPIs in yeast cells and other biological models.

In this sense, the integration of biological data in PPPI networks or graphs can be used to predict different cellular behaviors under specific physiological conditions [15]. Most of these PPPI networks are characterized by a power-law degree distribution, with a majority of the nodes or vertices having only a few links (also called edges) and a few nodes showing a very large number of edges [16]. Using the PPPI data available for the yeast *S. cerevisiae*, we evaluated if DNA repair proteins can interact with enzymes belonging to different Ca^{2+} homeostasis to repair and/or prevent damage induced by genotoxic agents like 4-NQO. For this purpose, an initial large binary PPPI network containing 4,399 yeast proteins (nodes) and 34,630 interactions (edges) was employed. The search for interactions among DNA repair proteins and enzymes of the glucose/fructose metabolic pathways was also performed with cPath [21]. This first screening generated binary PPPI subnetworks (data not shown) that were further refined using the iHOP software, which searches protein-protein interactions from the literature and proteomic databases [22]. This systems biology approach generated a single PPPI network regarding the interaction between DNA repair and Ca^{2+} homeostasis -associated enzymes (Fig. 5). It is interesting to note from this PPPI network the presence of many proteins that act in Ca^{2+} homeostasis (e.g., Pmr1p; Fig. 5). Among all the interactions observed with DNA repair and Ca^{2+} homeostasis mechanisms, Pmr1p connects directly with Rad4p, a protein that recognizes and binds to DNA lesions in association with Rad23p. Rad23p binds to Rad4p and stimulates activity in bind damage [22]. It can be observed that Rad23p is also present in the PPPI network, compounding a module together Rad33p [21] and Rad34p [23], whose functions in NER pathway are partially known. In this sense, it was described that Rad33p can be an additional factor affecting damage recognition by the Rad4p-Rad23p complex [24].

In addition, it can also observed in this PPPI network the interaction between Por1p and Pmr1p, showing a probable relationship of Ca^{2+} homeostasis systems between GC and mitochondria (Fig. 5). By its turn, Cod1p/Spf1p interacts with Hrd3p, an ER-associated enzyme associated to the degradation of 3-hydroxi-3-methylglutaryl-Coa reductase (Fig. 5). Thus, considering all the associations observed between Ca^{2+} homeostasis-associated enzymes and proteins involved in DNA repair/general metabolism in the PPPI networks, a gene ontology analysis was applied to determine the specific Ca^{2+} modulation of biological processes associated with repair/protection of the genome against genotoxic lesions.

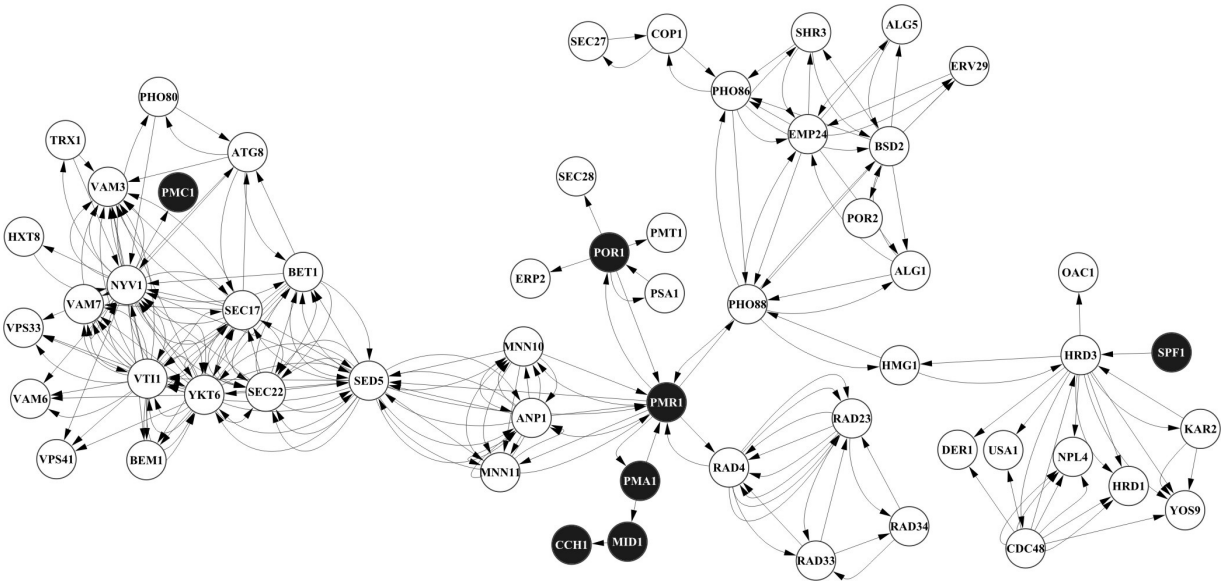

Fig. 5. A physical interaction network between Ca^{2+} homeostasis proteins (black circle), DNA repair and other metabolic process (white circle) obtained from global analyzes data banks of *S. cerevisiae* proteical interaction.

3.2 Gene ontology of Ca^{2+} homeostasis and DNA repair network

When gene ontology analysis was applied to the PPPI network, 12 major gene ontology biological categories were determined, mostly related to secretory mechanisms,

transport between organelles (like ER and GC), protein catabolism, protein glycosilation, Ca^{2+} ion transport an DNA repair by NER pathway (Table 3). Thus, considering this systems biology data, we initiate an experimental approach in order to validate the interactions observed.

Table 3. Mainly gene ontology classes (GO) obtained from interaction between Ca^{2+} homeostasis, DNA repair and other metabolic process.

Biologic process	**GO Clasify**	***p*[a] value**	**Corrected *p* value[b]**	***k*[c]**	***f*[d]**
Secretion way	45045	1.99×10^{-14}	2.21×10^{-12}	20	241
Vesicle Golgi Transport	48193	7.81×10^{-14}	3.58×10^{-12}	17	165
ER to Golgi transport by vesicles	6888	6.49×10^{-12}	1.98×10^{-10}	12	80
Protein catabolism process ER associate	30433	1.54×10^{-11}	4.24×10^{-10}	9	34
Protein catabolism process ubiquitin dependent	43161	4.86×10^{-9}	8.88×10^{-8}	9	62
Ion transport	6811	5.73×10^{-9}	9.27×10^{-8}	11	112
Protein glycosilation	6486	1.90×10^{-8}	2.48×10^{-7}	9	72
Ca^{2+} transport	6816	3.78×10^{-8}	4.45×10^{-7}	4	5
Retrograde transport by vesicle Golgi to ER	6890	7.00×10^{-8}	7.40×10^{-7}	6	24
Intra-golgi vesicle transport	6891	4.03×10^{-8}	3.83×10^{-7}	5	25
Nucleotide excision repair	6289	7.78×10^{-4}	4.50×10^{-3}	4	44
Damage recognition, NER	715	1.84×10^{-3}	9.56×10^{-3}	2	7

[a] p values calculated by hipergeometric distribution of one ontology class visualized in the network.
[b] Calculate values from *p* values after FDR applied.
[c] Total number of proteins find in the trap that belong a gene ontology.
[d] Total number of proteins that belong to a specific gene ontology.

3.3 Design of single and double yeast mutant strains deficient for Ca^{2+} homeostasis and *RAD4* gene and survival assay.

Taking into account the results obtained by System Biology analysis, it were generated single and double yeast mutants deficient for Ca^{2+} homeostasis and *RAD4* gene by single gene disruption. Once the yeast strains were tested against Ca^{2+} sensitivity in SynCo medium, we proceed to survival analysis applying the PQDT protocol [19].

The WT (BY4741; Table 1) strain, which is proficient in DNA repair and Ca^{2+} homeostasis, showed a higher survival in presence of different concentrations of 4-NQO when compared to the mutant yeast strains (Fig. 6). By its turn, all yeast mutants strains have a dose-dependent survival in culture media containing different concentrations of 4-NQO, specially the *cod1*Δ and *pmr1*Δ strains in the presence of 0.25 μM and 0.5 μM of 4-NQO, respectively (Fig. 6). Interestingly, the double mutant *pmr1*Δ*por1*Δ show a reduced survival when compared to the single mutant strains *pmr1*Δ and *por1*Δ for the same 4-NQO doses assayed (Fig. 6).

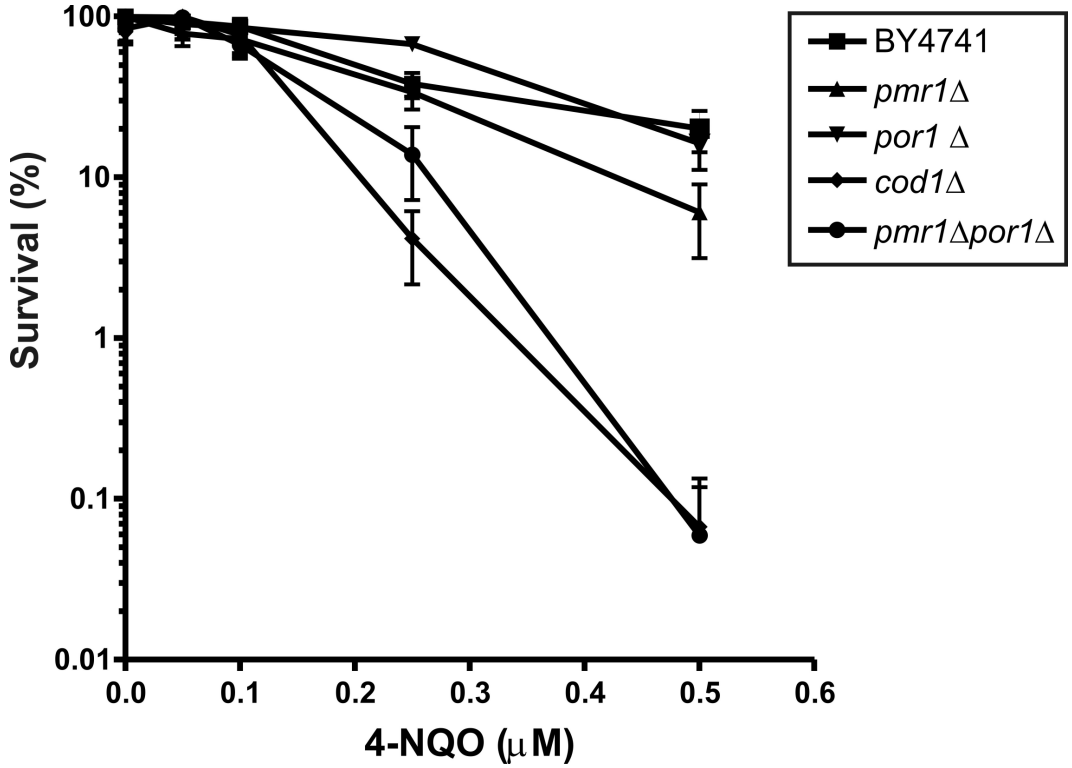

Fig. 6. Survival of *S. cerevisiae* single and double mutants deficient to Ca^{2+} homeostasis (*pmr1*Δ, *por1*Δ, *cod1*Δ e *pmr1*Δ*por1*Δ) after treatment with different 4-NQO concentration in SC solid media. The error bars represents the standard deviation for three separately treated cultures.

It is important to note that all survival assays performed *cod1*Δ mutant strains were realized in YEPD medium due to the lower survival of these strain in SC medium When single mutant *cod1*Δ was compared to the double mutants *cod1*Δ*por1*Δ and *cod1*Δ*pmr1*Δ it was possible to observe that *cod1*Δ is less sensitive (37% of survival when compared to WT strain). Moreover, it was observed a survival reduction in the double mutant strains *cod1*Δ*por1*Δ and *cod1*Δ*pmr1*Δ in the presence of high doses of 4-NQO (Fig. 7).

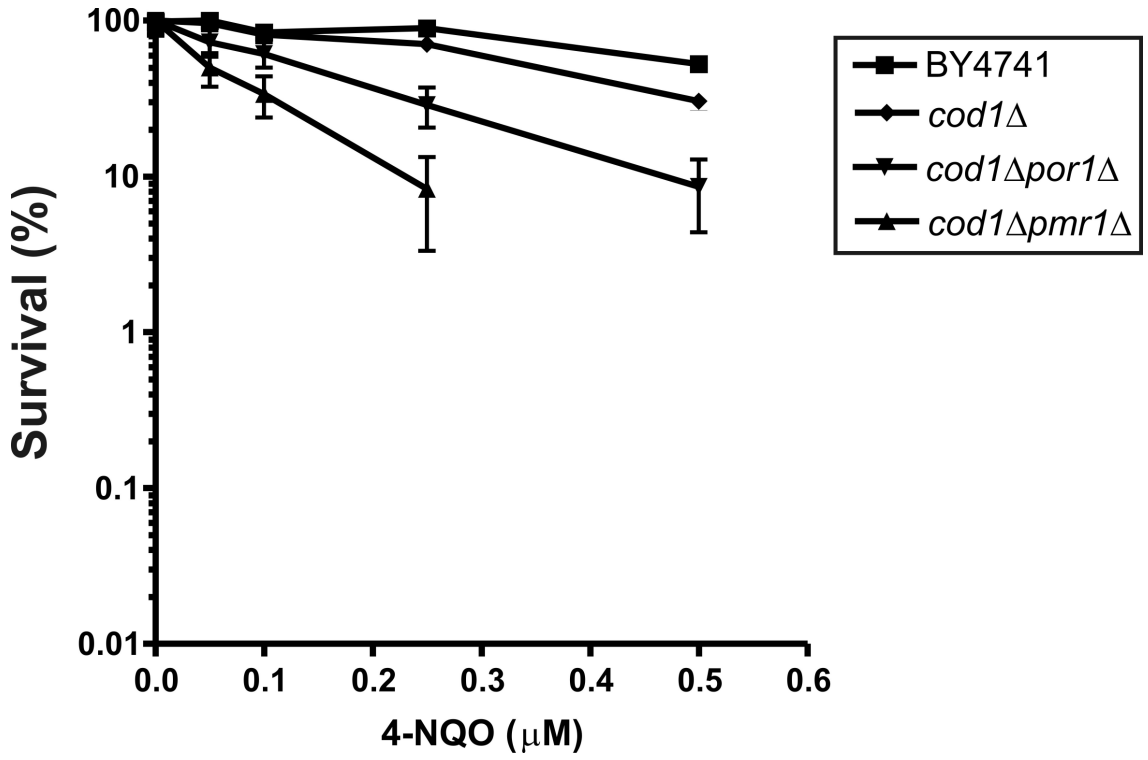

Fig. 7. Survival of *S. cerevisiae* single and double mutants deficient to Ca^{2+} homeostasis (*cod1*Δ, *cod1*Δ*por1*Δ e *pmr1*Δ*por1*Δ) after treatment with different 4-NQO concentration in YEPD solid media. The error bars represents the standard deviation for three separately treated cultures.

To proceed with the survival analysis of *rad4*Δ and associated double mutant strains, it was necessary to reduce the concentration of 4-NQO (0.5 nM to 10 nM) in the media due to the high sensitivity of these strains (Fig. 8A and 8B) In this sense, the *rad4*Δ mutant showed a reduced survival (39% of survival for 1 nM 4-NQO dose; Fig. 8A) when compared to the WT strain (76% of survival for 10 nM of 4-NQO dose; Fig. 8A). On the other hand, the double mutant *pmr1*Δ*rad4*Δ showed 27% of survival to 1 nM 0f 4-NQO dose, similar to the single *rad4*Δ mutant strain (Fig. 8A). In addition, the double mutant *cod1*Δ*rad4*Δ show a low survival when compared to the single mutant *rad4*Δ (Fig. 8B).

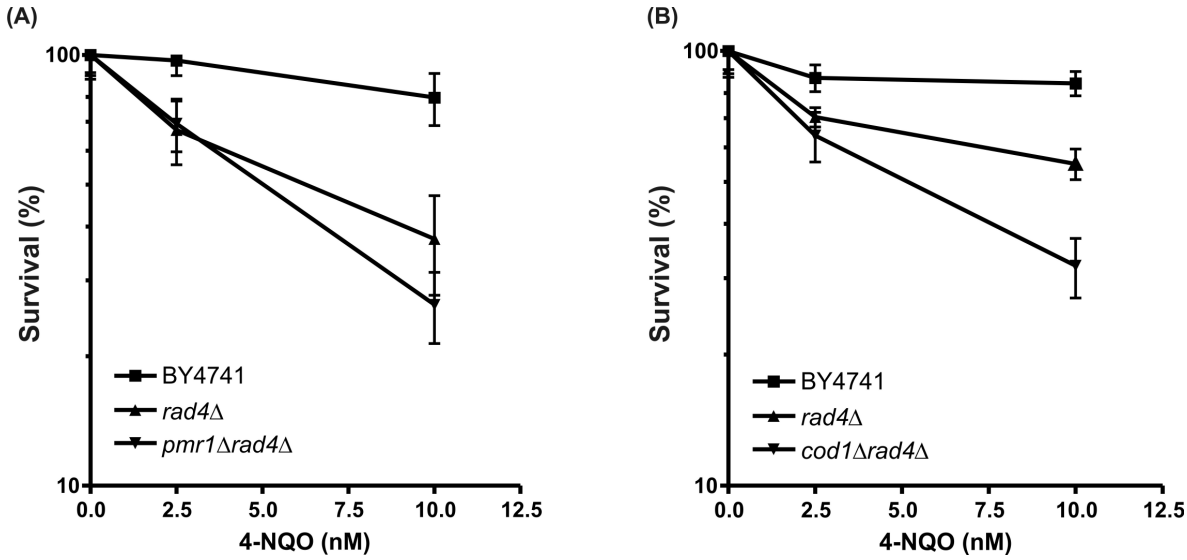

Fig. 8. In (A), *S. cerevisiae* single mutants survival deficients in NER way and double mutant to Ca^{2+} homeostasis and NER way repair (*rad4*Δ *e pmr1*Δ*rad4*Δ, respectively) after treatment with different concentrations of 4-NQO in SC solid media. In (B), *S. cerevisiae* single mutant deficient to NER way and double mutant to Ca^{2+} homeostasis and NER way repair (*rad4*Δ *e cod1*Δ*rad4*Δ, respectively) after treatment with different concentrations of 4-NQO in YEPD solid media. The error bars represents the standard deviation for three separately treated cultures.

3.4 Colony area measurement of *S. cerevisiae* strains proficient and deficient for Ca^{2+} homeostasis mechanisms and *RAD4*

In order to verify a possible cytostatic effect induced by 4-NQO in the different yeast strain tested the colony area was measured accordingly to the PQDT assay [19]. In this sense, it was possible to verify a reduction in the colony areas of WT, *pmr1*Δ, *por1*Δ, *cod1*Δ, and *pmr1*Δ*por1*Δ strains in SC medium containing different concentrations of 4-NQO (Table 4). In all yeast strains tested, the double mutant *pmr1*Δ*por1*Δ presents the smallest values of colony area (mm^2) (Table 4).

Table 4. Colony area measurement (mm^2) of *S. cerevisiae* strains BY4741, *pmr1*Δ, *por1*Δ, *cod1*Δ, and *pmr1*Δ*por1*Δ on SC solid media.

[4-NQO]	Yeast strains				
	BY4741	***pmr1*Δ**	***por1*Δ**	***cod1*Δ**	***pmr1*Δ*por1*Δ**
0 µM	2.44±0.16	2.14±0.13	2.08±0.45	2.02±0.081	0.79±0.015**
0.05 µM	2.09±0.45	2.03 ±0.13	0.91±0.58	1.46±0.14	0.70±0.081**
0.1 µM	1.57±0.24	1.38±0.023	0.46±0.045	1.26±0.32	0.43±0.070**
0.25 µM	1.15± 0.12	0.33±0.19	0.43±0.4	ND	ND
0.5 µM	0.49±0.14	ND	0.14±0.047	ND	ND

* $P \leq 0.05$ based on two-way ANOVA and the Dunnet's post-hoc test.

** $P \leq 0.01$ based on two-way ANOVA and the Dunnet's post-hoc test.

Abbreviation: not determined

By its turn, when the colony area of WT, *cod1*Δ, *cod1*Δ*por1*Δ, and *cod1*Δ*pmr1*Δ strains were measured in YEPD medium containing different concentrations of 4-NQO (Table 5), it was possible to verify that the *cod1*Δ mutant presents the smallest colony area when was compared to WT strain. Additionally, the *cod1*Δ*por1*Δ and *cod1*Δ*pmr1*Δ strains presents a reduction in the colony area, but only *cod1*Δ*pmr1*Δ presents a statistical difference ($P \leq 0{,}01$) when compared to WT strain.

The *rad4*Δ showed a colony area bigger than the WT strain in SC and YEPD media, respectively (Tables 6 and 7). The double mutant *pmr1*Δ*rad4*Δ showed a reduction in colony area, however, this difference was not statistically significant. On the other hand, the double mutant *cod1*Δ*rad4*Δ showed a reduction in the colony area when compared to the WT strain, being statistically significant ($P \leq 0{,}05$).

Table 5. Colony area measurement (mm^2) of *S. cerevisiae* strains BY4741, *cod1*Δ, *cod1*Δ*por1*Δ, and *cod1*Δ*pmr1*Δ on YEPD solid media.

[4-NQO]	Yeast strains			
	BY4741	***cod1Δ***	***cod1pmr1Δ***	***cod1por1Δ***
0 µM	2.34±0.12	2.73±0.11	1.01±0.31**	1.56±0.10
0.05 µM	2.15±0.11	2.41±0.05	0.17±0.09**	1.35±0.11
0.1 µM	1.91±0.14	2.30±0.07	0.17±0.07**	1.15±0.13
0.25 µM	1.44±0.11	2.46±0.09	ND	0.39±0.10
0.5 µM	0.75±0.08	1.69±0.14	ND	ND

* $P \leq 0.05$ based on two-way ANOVA and the Dunnet's post-hoc test.

** $P \leq 0.01$ based on two-way ANOVA and the Dunnet's post-hoc test..

Abbreviation: not determined

Table 6. Colony area measurement (mm^2) of *S. cerevisiae* strains BY4741, *rad4*Δ, and *pmr1*Δ*rad4*Δ on SC solid media.

[4-NQO]	Yeast strains		
	BY4741	***rad4Δ***	***pmr1Δrad4Δ***
0 nM	1.69±0.11	2.87±0.10	2.51±0.4
0.5 nM	1.53±0.27	1.55±0.05	2.02±0.7
1 nM	1.44±0.20	1.54±0.05	1.26±0.21
2.5 nM	1.40±0.19	1.44±0.14	0.99±0.06
10 nM	1.18±0.17	1.06±0.09	ND

Abbreviation: not determined

Table 7. Colony area measurement (mm^2) of *S. cerevisiae* strains BY4741, *rad4*Δ, and *cod1*Δ*rad4*Δ on YEPD solid media.

[4-NQO]	Yeast strains		
	BY4741	***rad4*Δ**	***cod1*Δ*rad4*Δ**
0 nM	2.23±0.12	2.05±0.07	2.44±0.14*
0.5 nM	2.18±0.10	2.64±0.09	2.05±0.11*
1 nM	2.15±0.05	2.64±0.13	2.02±0.14*
2.5 nM	2.14±0.11	2.61±0.10	1.51±0.16*
10 nM	1.74±0.08	2.51±0.22	0.83±0.24*

* $P \leq 0.05$ based on two-way ANOVA and the Dunnet's post-hoc test.

** $P \leq 0.01$ based on two-way ANOVA and the Dunnet's post-hoc test.

3.5 Cell cycle arrest assay in *S. cerevisiae* strains

Considering that citostatic effect of a chemical agent could be an indicative of cell cycle arrest, we performed an analysis of cell cycle arrest in the different *S. cerevisiae* strains used in this work. In this sense, the results indicated that the WT, *por1*Δ and *pmr1*Δ*por1*Δ strains shown cells majoritary in G1 phase for all conditions tested (Fig. 9A, 9C, and 9D).

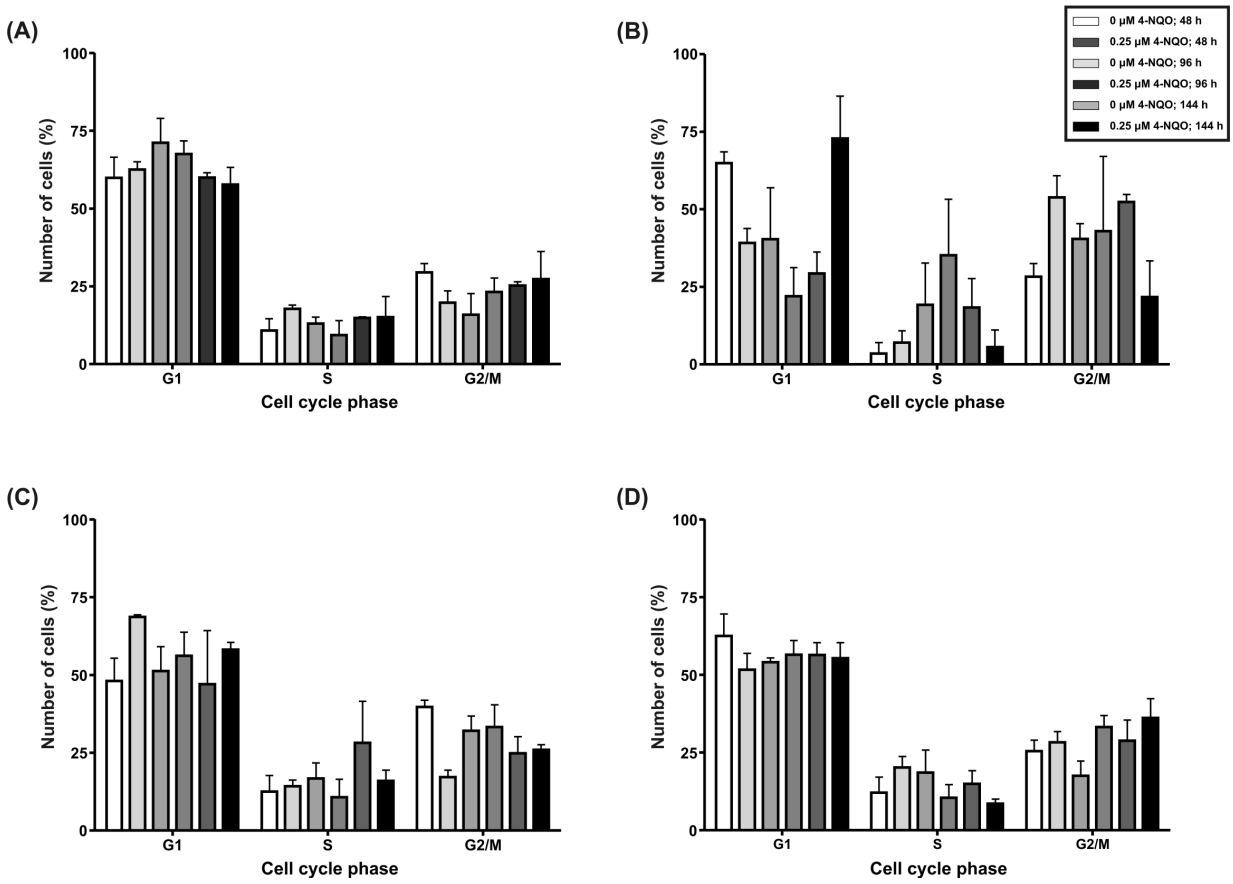

Fig. 9. Cell cycle arrest analysis of different *S. cerevisiae* strains (A, BY4741; B, *pmr1*Δ; C, *por1*Δ; D, *pmr1*Δ*por1*Δ). Treatment conditions are described in the box on the upper right.

However, the same behavior was not observed for *pmr1*Δ mutant, whose cells arrested in the G_2/M phase of cell cycle, except after 144 h in the presence of 0.25 μM of 4-NQO (Fig. 9B). It is interesting to note that *cod1*Δ (Fig. 10B) and *cod1*Δ*por1*Δ (Fig. 10D) arrest in the G_1 phase of cell cycle with exception of double mutant *cod1*Δ*pmr1*Δ, which arrest at G_2/M phase as the same way of single mutant *pmr1*Δ after 144 h in 0.25 μM of 4-NQO (Fig. 10). It should be noted that the disruption of both *COD1* and *RAD4* gene in the double mutant *cod1*Δ*rad4*Δ also results in cell cycle arrest at the G_2/M phase, while the

other gene combinations with *RAD4* gene disruption followed the normal cell cycle arrest at G_1 phase (Fig. 11).

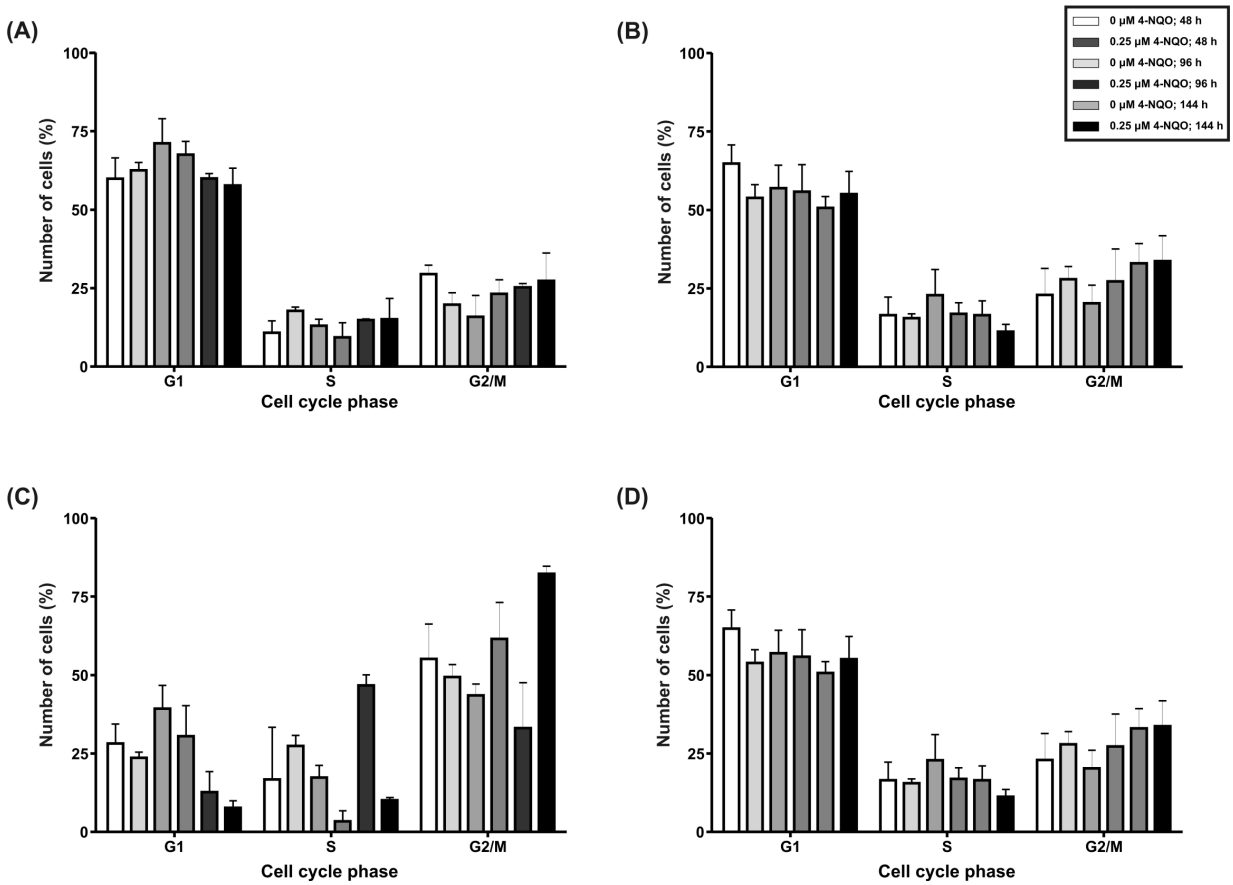

Fig. 10. Cell cycle arrest analysis of different *S. cerevisiae* strains (A, BY4741; B, *cod1*Δ; C, *cod1*Δ*pmr1*Δ; D, *cod1*Δ*por1*Δ). Treatment conditions are described in the box on the upper right.

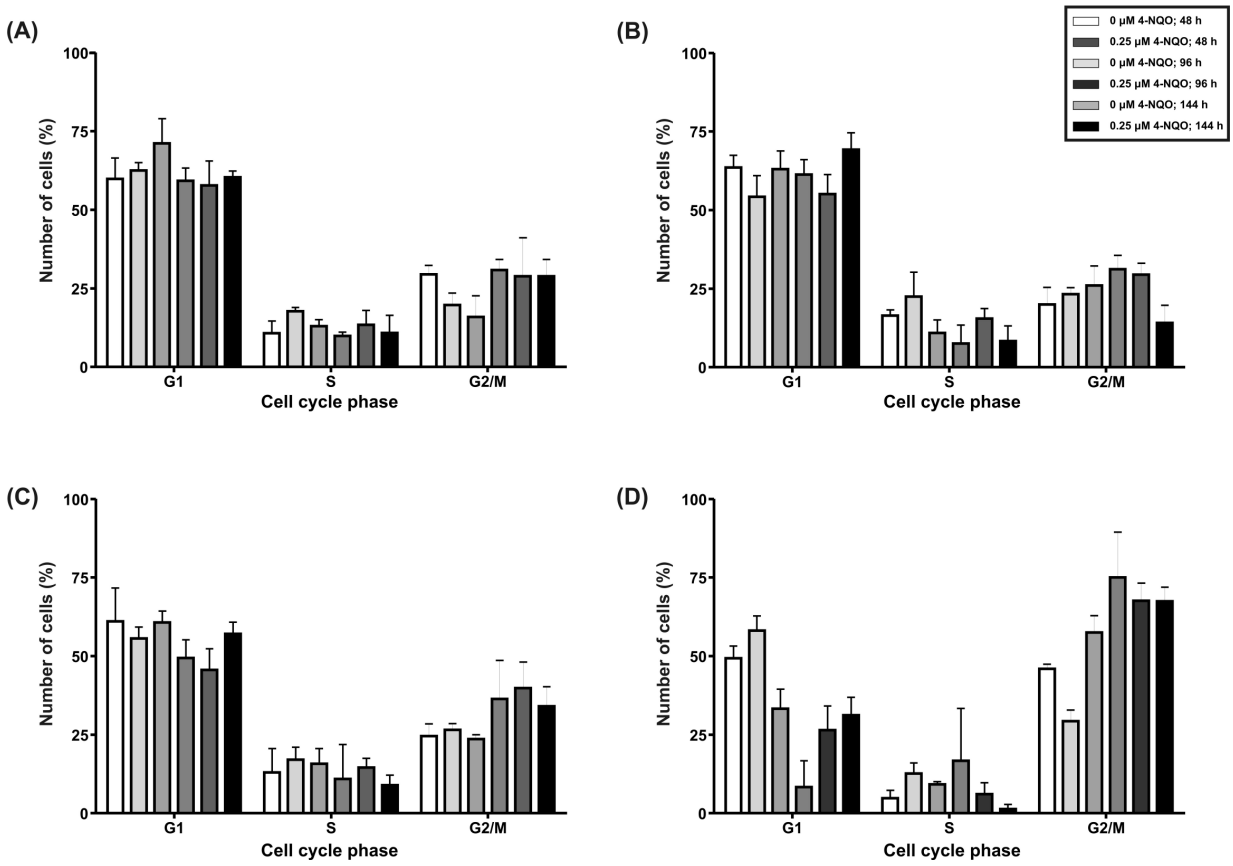

Fig. 11. Cell cycle arrest or delay assay in different *S. cerevisiae* strains (A, BY4741; B, *rad4*Δ; C, *pmr1*Δ*rad4*Δ; D, *cod1*Δ*rad4*Δ). Treatment conditions are described in the box on the upper right.

4. Discussion

The intracellular calcium concentration $[Ca^{2+}]_i$ in yeast and other fungi cells are normally regulated by a small set of Ca^{2+} transporters present in the cytoplasmic membrane or in membranous organelles. It has been related that mutations in any of these transporters can affect the $[Ca^{2+}]_i$ homeostasis, thus reducing the viability of *S. cerevisiae* cells under stress conditions [25]. Moreover, it also has been related that Ca^{2+} itself can activate DNA repair mechanisms after damage induced by reactive oxygen species (ROS), UVC, and

ionizing radiation [12]. A study using mononuclear cells treated with genistein, a calcium chelator, showed 40% reduction in DNA repair after damage induced by H_2O_2, suggesting that Ca^{2+} signaling is involved in oxidative DNA damage repair [26].

The results observed in our work corroborate the relationships between Ca^{2+} homeostasis and DNA repair mechanisms. The initial prospection for a possible Ca^{2+} homeostasis and DNA repair mechanisms interaction by a System Biology approach indicated that the Rad4p, a nucleotide excision repair protein, interacts with Pmr1p (Fig. 1), a high affinity Ca^{2+}/Mn^{2+} P-type ATPase required for Ca^{2+} and Mn^{2+} transport into Golgi complex. In this sense, it has been shown that Rad4p acts together with Rad23p to make a pre-complex that attracts the TFIIH holoenzyme to the site of lesion and initiate the repair by excision of damage strand [27]. Other proteins related to Ca^{2+} homeostasis are not directly linked to DNA repair, but control some aspects of cellular metabolism that appears to be important against genotoxic agents, like protein catabolism, secretion and intraorganellar transport (Table 3). Considering these results, we tested the yeast single and double mutants for Ca^{2+} homeostasis and *RAD4* against 4-NQO, a genotoxic agent that induces purine adducts [28] in a similar way of UVC radiation. Interestingly, the purine adducts induced by both 4-NQO and UVC are repaired by the same NER mechanisms [29].

An initial analysis of survival of single mutant cells for Ca^{2+} homeostasis mechanisms showed that only the *pmr1*Δ and *cod1*Δ strains mutants had a survival reduction in comparison to BY4741 (WT) after 4-NQO treatment (Fig. 6 and 7). This lowest survival for *pmr1*Δ and *cod1*Δ, as well as for double mutant *pmr1*Δ*cod1*Δ show the importance of these proteins in Ca^{2+} homeostasis and cell survival against genotoxic damages as expected from systems biology analyses. In this sense, the correlation between Ca^{2+} homeostasis and DNA repair were also observed with the double mutants *cod1*Δ*rad4*Δ

and *pmr1*Δ*rad4*Δ, where the interaction of these mutations is synergistic, resulting in a much lower survival when compared to the respective single mutant strains (Fig. 4A and 4B). Interestingly, the single mutant *por1*Δ, which is defective for Por1p, a mitochondrial voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability [30], did not showed sensitivity to 4-NQO when compared to the WT cell (Fig. 6), while a synergisitc interaction could be observed for the double mutant *cod1*Δ*por1*Δ (Fig. 7), indicating that the deletion of both genes affects the cellular response against 4-NQO.

One important aspect of the cellular answer against DNA damage induced by 4-NQO in *S. cerevisiae* is the strongest regulation and activation of DNA-damage sensing and cell cycle arrest mechanisms by different proteins [31]. Taking into account this idea, we evaluated if the single and double yeast mutant cells show cell cycle arrest after 4-NQO treatment. The data gathered from PQDT assay indicated that the single mutant *pmr1*Δ (Fig. 9B) and the double mutants *cod1*Δ*pmr1*Δ (Fig. 10D) and *cod1*Δ*rad4*Δ (Fig. 11D) have an increase in the numbers of cells in G_2/M phase after 4-NQO treatment. By its turn, all other yeast strains (including the WT strain) showed a G_1 phase arrest with or without drug treatment, an expected behavior in our assay condition. Supporting these findings, some genetic studies already suggested the Ca^{2+} involvement in the control of all phases of cell cycle except at the start of DNA synthesis [32]. Moreover, it has been related that the release of Ca^{2+} from internal storages (e.g., ER) is an important step for all mitotic-correlated events [33], and this fact could explain why all yeast strains deleted for *PMR1* and *COD1* gene in this work showed a cell cycle arrest at G_2/M phase. Another fundamental aspect of cell cycle checkpoints is its connection with DNA damage. Once a checkpoint has been activated by DNA damage, the DNA repair mechanisms are recruited.

However, if a DNA molecule can not be repaired appropriately, the cell goes to a permanently cell cycle arrest followed by apoptosis [34]. A second explanation that could be raised for the increased sensitivity of yeast *cod1*Δ and *pmr1*Δ mutant strains is related to ER stress. Considering that ER is the major site for protein synthesis and post-translational processing, and is responsible for correct protein folding, glycosylation, assembly of protein complexes, and the transport for the Golgi apparatus, it should be expected that any functional alteration in Cod1p and Pmr1p could also affect the functions of ER. In fact, it has been showed that the *pmr1*Δ mutants have a 50% reduction of Ca^{2+} in the ER [26, 35] resulting in the accumulation of misfolded proteins in the ER and causing ER stress [36]. In yeast, ER stress results in the stimulation of the Cch1p-Mid1p Ca^{2+} channel found in the plasma membrane of cells, elevating the cytosolic free Ca^{2+} and activating the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin [37]. Once activated, calcineurin can delay the transition of G_2 to M phase during the cell division by increasing the phosphorylation of Cdc28, a protein that is the catalytic subunit of the main cell cycle cyclin-dependent kinase (CDK). Another fact that can explain the increased sensitivity of *cod1*Δ and *pmr1*Δ mutant to 4-NQO, considering the ER stress mechanism, is the fact that Rad23p, a protein with ubiquitin-like N terminus that recognizes and binds damaged DNA together Rad4p during NER and also detected in our PPPI network (Fig. 5), also participate in the degradation of proteins in ER, being translocated to the 26S proteasome towards the ER-membrane [38]

In conclusion, we proposed that Ca^{2+} homeostasis proteins, especially Cod1p and Pmr1p, can affect the DNA-damage response induced by 4-NQO. The action of Cod1p and Pmr1p can be related to the activation and its interaction with the NER-associated protein Rad4p as observed by systems biology and experimental data. On the other hand, it is

possible *cod1*Δ and *pmr1*Δ mutation also results in the generation of an ER stress that negatively affects the cells' response against 4-NQO. Additional biochemical experiments are being conduced in role to elucidate the role of Cod1p and Pmr1p in the DNA damage responses.

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DISCUSÃO GERAL

Já foi descrito na literatura que o íon Ca^{+2} desempenha importante papel na fisiologia das células e que a manutenção de sua concentração em níveis constantes se faz necessária para a manutenção da viabilidade celular. Esta concentração, entretanto, é mantida por proteínas, as quais possuem muita similaridade entre mamíferos e leveduras (Santella & Carafoli, 1997).

Além da importância da manutenção da viabilidade celular, o desequilíbrio da homeostase do Ca^{+2} em seres humanos está relacionado a inúmeras patologias, mas principalmente com doenças neurodegenerativas (Trump and Berezeski, 1995). Alguns estudos também já correlacionaram o íon Ca^{+2} com a ativação e controle de proteínas relacionadas com os mecanismos de resposta a danos celulares (Trump e Berezeski, 1995; Gafter *et al*., 1997; Ahlers *et al.*, 1999), onde há um aumento na concentração intracelular deste íon após uma determinada injúria. Este aumento do influxo de Ca^{+2} gerado pela exposição ao agente estressor também pode levar à apoptose, conforme visto no Capítulo I desta dissertação (McConey and Orrenius, 1996).

A relação existente entre os mecanismos de homeostase do Ca^{+2} e as deficiências nos mecanismos de reparação de danos no DNA vem sendo demonstrada principalmente nas doenças neurodegenerativas como Alzheimer, Parkinson e Esclerose Amiotrófica Lateral onde a deficiência da resposta da estimulação do Ca^{+2} está relacionada com deficiências nos mecanismos de reparação dos danos.

Tendo em vista a importância tanto da homeostase da célula e dos mecanismos de reparação de DNA buscou-se, neste trabalho, compreender as vias de interação e as proteínas envolvidas nestes processos. Para este fim realizou-se uma compilação de dados

já existentes na literatura que pudessem estabelecer algumas interações a este respeito (Capítulo I), o que nos permitiu um direcionamento para as descobertas posteriores.

Em função disso, utilizando-se ferramentas de Biologias de Sistemas, foi possível gerar uma rede de interação proteína-proteína, a qual possibilitou a visualização da interação entre as proteínas de homeostase de Ca^{+2} e algumas proteínas envolvidas no mecanismo de reparação de DNA da via NER (Capítulo III). Esta rede de interação mostra que a proteína Pmr1p (do complexo de golgi) exerce interação direta, além de outras proteínas, com a proteína Rad4p e esta, por sua vez, interage com as proteínas Rad23p, Rad33p e Rad34p. A proteína Pmr1p também exerce influência sobre Por1p (da membrana mitocondrial) com as proteínas de homeostase de Ca^{+2} presentes na membrana plasmática e com a proteína Cod1p (do retículo endoplasmático).

Com a utilização de ferramentas de Biologia de Sistemas foi possível também realizar uma análise ontológica dos genes que codificam para as proteínas destacadas na rede de interação (Capítulo III). Com isso foi possível correlacionar estas proteínas com processos biológicos importantes para as células e organelas envolvidas na manutenção dos níveis de Ca^{+2} intracelular. Estes processos envolvem transporte de íons, de vesículas entre organelas, catabolismo e glicosilação de proteínas, o reparo e o reconhecimento de danos no DNA pela via NER, dentre outros.

Frente a estas informações obtidas a partir da análise de Biologia de Sistemas, linhagens de *S. cerevisiae* deficientes de proteínas de homeostase de Ca^{+2} e no gene *RAD4* foram construídas e foram conduzidos estudos de citotoxicidade, citostaticidade e parada de ciclo celular após exposição ao agente genotóxico 4-NQO (Capítulo III).

As proteínas responsáveis pela homeostase de Ca^{+2} e que parecem estar envolvidas com a via NER são a Pmr1p e a Cod1p (Capítulo III). Um estudo realizado por Strayle *et*

al. (1999) mostrou que mutantes *pmr1Δ* apresentam uma redução de até 50% na concentração de Ca^{+2} no interior do RE, comprovando que a proteína Pmr1p também exerce efeito sobre o RE. Isto também indica que esta organela possui papel importante na manutenção dos níveis de Ca^{+2} e também na resposta aos danos no DNA.

Sabe-se que o RE é o compartimento celular para o processamento de proteínas como empacotamento, glicosilação, produção de complexos de proteínas e transporte para o CG. Sendo esta organela a que contém o maior estoque de Ca^{+2} da célula e é responsável pela liberação deste íon para o citosol mediante resposta a um estímulo (Kaufman 1999), estima-se que sua concentração interna também deva ser mantida para o bom funcionamento de suas atividade. Neste sentido, um desequilíbrio na homeostase do Ca^{+2} celular como resposta a um estímulo pode levar ao estresse de retículo que, por sua vez, pode ser seguido de alterações como: (i) acúmulo de proteínas não dobradas ou mal dobradas, (ii) alterações no metabolismo da glicose e (iii) colesterol e estresse oxidativo. Nesse sentido, estas alterações podem levar a inibição da síntese de proteínas e apoptose celular (Fathallah-Shaykh, 2005). Esta inibição da síntese de proteínas pode envolver proteínas importantes tanto para o reconhecimento quanto para a reparação do dano e se este não for reparado eficientemente a célula poderá evoluir para a apoptose.

Na levedura *S. cerevisiae* a homeostase de Ca^{+2} é feita por proteínas situadas na membrana de organelas (Cod1p, Pmr1p, Pmc1p, Por1p), na membrana citoplasmática (Cch1p, Mid1p) e proteínas citoplasmáticas (calmodulina e calcineurina). A deficiência de proteínas da homeostase de Ca^{+2}, principalmente Pmr1p e Cod1p, eleva os níveis deste íon no citoplasma ativando outras vias de manutenção de homeostase, como a da calmodulina e da calcineurina. Estas duas porteínas podem ativar os mecanismos de parada de ciclo

celular. Se o dano não for reparado, os mecanismos de indução de morte celular poderão, por sua vez, ser ativados (van Laar *et al*., 2002; Bonilla *et al*., 2003).

Os dados da literatura indicam que o Ca^{+2} exerce influências sobre a reparação o DNA, mas nossos resultados (Capítulo III) mostram que esta influência afeta a via de reparação do NER e a função da proteína Rad4p, que faz o reconhecimento da lesão e auxilia na pré-incisão deste dano (Wood, 1997). Outro fator importante é que a interação com a proteína Rad4p afeta outras proteínas, como a Rad23p que, em conjunto com Rad4p (Wood, 1997), reconhecem e se ligam ao dano, e também a outras proteínas, como Rad33p (Hanway *et al*., 2002) e Rad34p (Huh *et al*., 2003), cujas atividades ainda não estão esclarecidas na literatura.

Adicionalmente, os resultados de citotoxicidade e de citostaticidade (Capítulo III) das linhagens deficientes nos genes que codificam para proteínas responsáveis pela homeostase de Ca^{+2} denotam a importância das proteínas Pmr1p e Cod1p frente aos danos causados pelo agente genotóxico 4-NQO. Estes achados também elucidam que o RE, e conseqüentemente, o CG possuem um papel importante na célula frente aos danos gerados, pois a deficiência de proteínas que proporcionam a homeostase dentro destas organelas ou que mantenham as concentrações intracelulares de Ca^{+2} em níveis constantes afetam diretamente a sobrevivência celular. Isto está diretamente relacionado com o estresse de retículo descrito anteriormente.

Os ensaios de parada de ciclo celular (Capítulo III) comprovam que, na ausência das proteínas Pmr1p e Cod1p a célula fica incapacitada de reparar os danos, o que resulta em uma parada de ciclo celular que pode evoluir para a apoptose. Isto pode estar correlacionado com a ativação de outras vias como a da calmodulina e calcineurina que

estão relacionados com os mecanismos de parada de ciclo celular (Bonilla e Cunningham, 2003).

Para os estudos de citotoxicidade e citosticidade realizados nesta dissertação, desenvolvemos uma técnica chamada de PQDT (Capítulo II). Esta técnica permite observar efeitos citotóxicos e citostáticos de linhagens deficientes e proficientes em determinados genes frente à exposição a um agente genotóxico. Nossos resultados, avaliando os efeitos dos agentes estressores 4-NQO e MMS e do agente antitumoral 5-FU, mostraram que esta metodologia permite um estudo simultâneo dos efeitos citotóxicos e citostáticos dos agentes testados. Além disso, esta técnica pode ser adaptada para estudos farmacocinéticos, sendo possível prospectar vias bioquímicas importantes e até mesmo desvendar mecanismos de ação de agentes genotóxicos ou de fármacos ainda desconhecidos (Capítulo II). Assim, esta metodologia se mostra uma técnica de execução simples, com menor risco de contaminação das culturas, utilizando maiores volumes de suspensões celulares e minimizando os erros de medida volumétrica, impedindo a resposta celular à falta de nutrientes e desenvolvimento de resistência celular, como também não necessitando de equipamentos sofisticados, podendo ser aplicado facilmente em qualquer laboratório que já utilize a levedura *S. cerevisiae* para ensaios celulares, bioquímicos e moleculares.

CONCLUSÃO GERAL

As proteínas responsáveis pelos mecanismos de homeostase de cálcio e pelos mecanismos de reparação de DNA apresentam correlação importante, principalmente para a via NER. A análise de linhagens de levedura deficientes para determinadas proteínas permitiu uma correlação mais estreita existente entre as proteínas Cod1p, Pmr1p e Rad4p. Isso nos permitiu inferir que a sobrevivência reduzida dos simples mutantes e duplos mutantes para estas proteínas é devida, principalmente, ao estresse de retículo causado pelo desequilíbrio da $[Ca^{+2}]i$. E, além disso, a utilização das ferramentas de Biologia de Sistemas permitiu um delineamento e um direcionamento dos estudos de interações proteícas.

CONCLUSÕES ESPECÍFICAS

- Levando-se em conta os resultados obtidos na análise de Biologia de Sistemas, a homeostase de Ca^{+2} exerce influência sobre os mecanismos de reparação de DNA, principalmente nas proteínas da via NER, como a Rad4p.
- As proteínas Cod1p e Pmr1p são as proteínas de homeostase de Ca^{+2} mais importantes na resposta ao dano celular, devido à baixa sobrevivência apresentada após exposição ao agente 4-NQO. E essa resposta se torna ainda mais reduzida quando a deficiência destas proteínas é combinada com a mutação para o gene *RAD4*.

- Portanto, o estresse de retículo e a ativação de outras vias que levam à parada de ciclo celular e foram os principais processos metabólicos evidenciados nas linhagens de leveduras testadas, após a exposição ao agente genotóxico 4-NQO.
- Verificou-se também, pela análise de interação proteína-proteína e análise ontológicas dos genes envolvidos, que o Ca^{+2} exerce influência em inúmeras vias importantes dentro da célula e que os efeitos do desequilíbrio da sua homeostase não se restringem à deficiências na reparação de danos no DNA, mas a processos que também podem influenciar na manutenção da viabilidade celular.

PERSPECTIVAS

A análise de Biologia de Sistemas, seguida de ontologia gênica, revelou uma ampla rede de interações de proteínas e vias metabólicas importantes onde muitas destas proteínas possuem atividades que ainda não foram estabelecidas ou não estão bem elucidadas. Assim, propõem-se alguns estudos que podem ser realizados para ampliar os conhecimentos de interação entre todas estas proteínas:

- Verificar a influência da proteína HMG1 na homeostase de Ca^{+2} e sua relação com Pmr1p e Cod1p, frente às atividades bioquímicas e fisiológicas que desempenham por meio de ensaios *in vitro* utilizando linhagens mutantes para estas proteínas.
- Sabendo-se que as proteínas de membrana citoplasmática Cch1p e Mid1p desempenham também papel no estresse de retículo, avaliar o comportamento de linhagens deficientes para os genes que codificam para estas proteínas frente à exposição de agentes genotóxicos.
- Correlacionar a interação existente entre Pmr1p e as proteínas do NER Rad33p e Rad34p.
- Como os resultados obtidos neste trabalho se correlacionam com a via NER e os efeitos citotóxicos do 4-NQO, realizar ensaios com agentes genotóxicos de mecanismos de ação diferentes e que possam ser reparados pelas mais diversas vias de reparação, verificando a influência da homeostase de Ca^{+2} com estes outros mecanismos.

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