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RESEARCH

Autophagy and apoptosis in the oxidative stress protection of extracellular acidity

Ekstraselüler asiditenin oksidatif strese karşı koruyuculuğunda otofaji ve apoptozun etkisi

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Abstract

Purpose: This study aimed to investigate the impact of extracellular acidity on oxidative stress resistance and to explore the interplay between apoptosis and autophagy on *Saccharomyces cerevisiae*.

Materials and Methods: Cells were cultured under either standard or acidic media and exposed to 8 mM hydrogen peroxide (H_2O_2) to induce oxidative stress. Cell viability, intracellular reactive oxygen species (ROS) levels, apoptotic cell ratio, and autophagolysosomes were evaluated by colony-forming unit (CFU) assay, fluorescence-based staining techniques, and microscopy. Mutant strains deficient in key genes in apoptosis and autophagy were utilized to evaluate specific contributions of these pathways to oxidative stress resistance.

Results: Cells cultured in the acidic environment showed resistance to oxidative stress. The CFU fold change ratios after 30 minutes of exposure were increased from 0.031 to 0.181, ROS levels were reduced from 18.35% to 2.44%, apoptotic cell ratios were decreased from 21.06% to 12.45%, and autophagic activities were increased from 97.68 to 115.48. In mutant strains subjected to oxidative stress in acidic media, CFU fold change ratios were also higher in *yca1*Δ (0.172), *atg4*Δ (0.215), and *atg8*Δ (0.153) compared to wild-type cells (0.103), indicating that both apoptosis and autophagy pathways contribute to the oxidative stress response under acidic conditions.

Conclusion: These findings provide novel insights into the influence of acidic microenvironments on cellular stress responses and may contribute to the development of therapeutic strategies for diseases associated with oxidative damage and tissue acidosis.

Keywords: apoptosis; extracellular acidity; oxidative stress; autophagy; *Saccharomyces cerevisiae*

Öz

Amaç: Bu çalışmada, ekstraselüler asiditenin oksidatif stres direncine ve hücre ölüm yolaklarına etkileri *Saccharomyces cerevisiae* modelinde ilk kez araştırılmıştır.

Gereç ve Yöntem: *S. cerevisiae* hücreleri normal ve asidik pH'a sahip ortamlarda kültüre edilerek 8 mM hidrojen peroksit (H_2O_2) ile oksidatif strese maruz bırakıldı. Hücre canlılığı, reaktif oksijen türleri (ROS) birikimi, apoptotik hücre oranları ve otofajik aktivite, CFU sayımı, floresan boyama ve mikroskopi yöntemleriyle değerlendirildi. Ayrıca, apoptoz ve otofaji ile ilişkili genetik mutantlar kullanılarak bu süreçlerin stres yanıtındaki rolleri araştırıldı.

Bulgular: Asidik ortamda bulunan hücreler, oksidatif strese karşı daha yüksek hayatta kalma oranı gösterdi. Bu durum, daha düşük ROS düzeyleri (%18,35'ten %2,44'e), daha düşük apoptoz oranları (%21,06'dan %12,45'e) ve artan MDC floresan oranı (97,68'den 115,48'e) ile doğrulandı. Ayrıca, 30 dakikalık H_2O_2 maruziyeti sonrasında CFU kat değişimi oranı, asidik koşullarda 0,031'den 0,181'e yükseldi. Asidik ortamda oksidatif strese maruz bırakılan mutant suşlarda CFU kat değişim oranları *yca1*Δ (0,172), *atg4*Δ (0,215) ve *atg8*Δ (0,153) suşlarında yabanıl tipe (0,103) kıyasla daha yüksek bulunmuştur. Bu bulgular, hem apoptoz hem de otofaji yolaklarının asidik koşullarda oksidatif stres yanıtına katkı sağladığını göstermektedir.

Sonuç: Bu bulgular, asidik mikroçevrenin hücre kaderini nasıl etkilediğine dair yeni bilgiler sunmakta olup, oksidatif stres ve doku asidozu ile ilişkili hastalıklarda tedavi stratejileri açısından önemli bir hedef olabileceğini göstermektedir.

Anahtar kelimeler: apoptoz; ekstraselüler asidite; oksidatif stres; otofaji; *Saccharomyces cerevisiae*

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INTRODUCTION

Extracellular acidity is a hallmark of malignant solid tumor microenvironments, primarily resulting from poor perfusion, hypoxia, and altered cellular metabolism[1]. Tumor cells frequently exhibit elevated glycolytic activity and lactic acid fermentation, leading to the excessive production of lactate and protons (H^+). Inadequate vascular perfusion limits the clearance of these acidic byproducts, contributing to the acidification of the tumor milieu[2]. This acidic microenvironment can influence the efficacy of chemotherapeutic agents through multiple mechanisms, including the upregulation of drug efflux pumps, activation of proton transporters, induction of the unfolded protein response, and stimulation of autophagy[3].

Acidic microenvironments do not occur solely in tumors; infected tissues can also undergo acidification due to the release of lactate and H^+ by activated neutrophils and other immune cells[4]. Exposure to low extracellular pH has been shown to induce a wide range of cellular effects. For example, it can reprogram macrophage metabolism[5], activate neutrophils and delay their apoptosis[6], enhance endothelial cell adhesion[7], and even inhibit endothelial cell apoptosis[8]. Notably, although elevated temperatures typically accelerate neutrophil apoptosis under neutral pH conditions, acidic environments have been reported to prolong neutrophil survival[9]. These findings collectively suggest that extracellular acidity may offer a broad cytoprotective effect under various stress conditions. However, its precise role in modulating oxidative stress sensitivity has yet to be clearly defined.

Saccharomyces cerevisiae is a well-established model organism for investigating cellular responses to stress and various forms of programmed cell death. Similar to mammalian cells, yeast can undergo apoptosis, necrosis, and autophagy-associated cell death, each characterized by distinct morphological and biochemical markers[10,11]. As in mammals, apoptosis in yeast involves both caspase-dependent and -independent pathways. In the caspase-dependent mechanism, the metacaspase Yca1 functions as the central executioner protease. Alternatively, caspase-independent apoptosis is mediated by mitochondrial factors such as apoptosis-inducing factor 1 (Aif1) and endonuclease G, which promote nuclear degradation and chromatin condensation independently of Yca1 activity[12]. In addition to apoptosis, yeast also undergoes autophagy, a conserved catabolic process activated under diverse stress conditions, such as nutrient deprivation, oxidative stress, or intracellular damage. This mechanism facilitates the degradation of damaged organelles and misfolded proteins via double-membraned autophagosomes and is tightly regulated by autophagy-related (Atg) proteins[13,14]. Although autophagy generally promotes cell survival, excessive or dysregulated activation can lead to autophagy-mediated cell death[15]. Moreover, apoptosis and autophagy share several regulatory components, suggesting a complex interplay between these pathways even in unicellular organisms such as yeast[16].

Similar to higher eukaryotes, *S. cerevisiae* adapts to acidic extracellular pH by modulating gene expression and activating stress response pathways[17]. Preconditioning yeast cells in acidic media has been shown to enhance their resistance to acetic acid–induced cell death[18,19]. However, the direct effects of extracellular acidity on apoptosis and autophagy in yeast remain poorly characterized[20,21], and its role in modulating oxidative stress-induced cell death is still not fully understood. Given the involvement of acidic pH in metabolic reprogramming, it may influence the regulation of cell death pathways, with potential implications for therapeutic strategies targeting diseases associated with oxidative stress.

While oxidative stress is a well-established trigger of regulated cell death, the impact of extracellular acidity on these responses remains largely unexplored. Considering the prevalence of acidic microenvironments in pathological conditions such as tumors and sites of inflammation, understanding how acidity affects oxidative damage is critically important. This study focuses on the immediate effects of acidic extracellular pH on hydrogen peroxide (H_2O_2)–induced oxidative stress in yeast.

We hypothesized that acidic pH modulates oxidative stress responses by differentially influencing apoptotic and autophagic processes. To investigate this, we first assessed the effects of extracellular acidity on cell viability, intracellular ROS levels, apoptotic cell ratios, and autophagic activity in wild-type *S. cerevisiae*. To further evaluate the specific contributions of apoptosis and autophagy, we examined oxidative stress sensitivity in mutant strains lacking key apoptotic (*yca1Δ, aif1Δ*) or autophagic (*atg4Δ, atg8Δ*) genes. These results shed light on the

role of acidic environments in cellular stress resistance and may help understanding similar mechanisms in higher organisms.

MATERIALS AND METHODS

Yeast strains, media, and culture conditions

This study was conducted under controlled laboratory conditions using the unicellular model organism *S. cerevisiae* to examine immediate cellular responses to extracellular acidity and oxidative stress. All experimental procedures were carried out at the Department of Molecular Biology and Genetics, Maltepe University (Istanbul, Türkiye), by a research team consisting of a molecular biologist, an assistant professor, and an associate professor. As the study did not involve human participants, animal models, or patient-derived materials, ethical approval was not required.

The following *S. cerevisiae* strains, all derived from the BY4741 genetic background, were used in this study: wild-type (WT) (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0), *yca1*⊿ (MATa; leu2Δ0; ura3Δ0; his3Δ0; met3Δ0; yca1::KANMX4), *aif1*⊿ (MATa; leu2Δ0; ura3Δ0; his3Δ0; met3Δ0; aif1::KANMX4), *atg4*⊿ (MATa; leu2Δ0; ura3Δ0; his3Δ0; met3Δ0; atg4::KANMX4), and *atg8*⊿ (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; atg8::KANMX4). All strains were streaked onto YPD agar plates containing 1% yeast extract, 2% peptone, 2% glucose, and 2% agar. After 2 days of incubation at 30 °C, a single colony from each strain was inoculated into liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose; pH ≈ 6.8) and incubated at 30 °C with shaking at 175 rpm until saturated cultures were obtained.

Exposure to acidic environment and induction of oxidative stress

Following overnight incubation in fresh YPD media, saturated cultures had grown to the late logarithmic phase (OD_{600} ≈ 1.0). Cultures were then divided into two equal aliquots and centrifuged. One aliquot was resuspended in standard YPD media (pH ≈ 6.8), while the other was resuspended in acidic YPD media (pH ≈ 3.5). Each was subsequently divided into two subcultures, and oxidative stress was induced by the addition of H_2O_2 to a final concentration of 8 mM.

Consequently**,** four experimental conditions were established for each strain: Regular pH – 0 mM H_2O_2, Regular pH – 8 mM H_2O_2, Acidic pH – 0 mM H_2O_2, and Acidic pH – 8 mM H_2O_2. These cultures were incubated at 30 °C with shaking at 175 rpm, and samples were collected at the indicated timepoints. All experiments were conducted in at least three independent biological replicates.

Determination of colony-forming units (CFU)

At each timepoint, 100 µL of culture was collected and subjected to a 10-fold serial dilution (10^{-1} to 10^{-5}). Appropriate dilutions were plated on YPD agar and incubated at 30 °C for two days. Colony-forming units (CFU) were counted, and CFU/mL values were calculated by multiplying the colony number by the corresponding dilution factor and dividing by the plated volume (0.1 mL). Fold changes in CFU/mL were determined by normalizing each value to that of the beginning sample[22]. Data from at least three independent biological replicates were presented as the mean ± standard error of the mean (SEM).

Detection of reactive oxygen species (ROS)

Intracellular ROS levels were quantified using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes), as previously described[23]. The cell-permeable probe H_2DCFDA is non-fluorescent until oxidized by ROS, which convert it into the highly fluorescent compound dichlorofluorescein (DCF)[24].

At each timepoint, 200 µL of each culture was collected and resuspended in fresh YPD medium. H_2DCFDA was added to a final concentration of 10 µg/mL. Samples were incubated in the dark at 30 °C for 40 minutes. Subsequently, 5 µL of each sample was placed on a slide and examined under a fluorescence microscope (Leica DM1000 LED, Germany). ,For each sample, at least 200 cells were analyzed and categorized as DCF-positive (fluorescent) or DCF-negative. The percentage of fluorescent cells was calculated, and the average from three independent replicates was reported as the mean ± standard deviation (SD).

Hoechst 33342/propidium iodide dual staining

Hoechst 33342 and propidium iodide (PI) dual staining was performed according to a previously established protocol[25]. Briefly, 100 µL of each culture

was collected, washed with, and resuspended in cold PBS. Hoechst 33342 and PI were added to the cell suspensions at final concentrations of 5 μg/mL and 1 μg/mL, respectively. After 20 minutes of incubation on ice, cells were analyzed by fluorescence microscopy (Leica DM1000 LED, Germany). A minimum of 200 cells per sample were counted and categorized as apoptotic ($Hoechst^+/PI^-$), late apoptotic or necrotic ($Hoechst^+/PI^+$), or live (non-fluorescent). Results from three replicates were reported as the mean ± SD.

Monodansylcadaverine (MDC) staining

Autophagic vacuoles were visualized using monodansylcadaverine (MDC), a fluorescent dye that accumulates in autophagolysosomes[26]. Cultures were centrifuged and fixed in 70% ethanol at room temperature for 30 minutes, washed, and resuspended in PBS. Cells were then incubated with 0.05 mM MDC in PBS for 10 minutes at 37 °C[27]. Next, cells were washed four times with PBS containing 0.1% Triton X-100 and 10 mM Tris-HCl, and resuspended in PBS.

A volume containing 1×10^5 cells per well was transferred to a black 96-well plate in triplicate. The final volume of each well was adjusted to 100 μL with PBS. Fluorescence was measured using a microplate reader (BioTek Instruments) at 335 nm excitation and 535 nm emission. Values were normalized to control samples and expressed as %MDC fluorescence (relative fluorescence units). Mean values from three independent experiments were reported as mean ± SD.

Statistical analysis

All experiments were performed in at least triplicate, and results are reported as mean ± SD or SEM. To compare group-level differences in non-normally distributed data, the non-parametric Kruskal–Wallis test was applied. This test was used for comparisons of CFU fold changes, ROS-positive cell percentages, apoptotic cell ratios, and MDC fluorescence intensities across multiple groups.

Test statistics (KW) and p-values were reported for each analysis. For pairwise comparisons between two groups, the Mann–Whitney U test was used.

Effect sizes were calculated using rank-biserial correlation (r), where r values of 0.1–0.3 were interpreted as small, 0.3–0.5 as moderate, and ≥0.5 as large effects. GraphPad Prism (v5) was used for all statistical analyses, and a p-value < 0.05 was considered statistically significant.

RESULTS

Acidity alone does not cause cell death

To investigate whether extracellular acidity affects the growth of S. cerevisiae, cell viability was monitored over a 3-hour period in YPD medium at either acidic or neutral pH (Figure 1). Colony-forming unit (CFU) fold changes were calculated at 10-minute intervals during the first hour and at 30-minute intervals thereafter.

Figure 1 shows a time-dependent increase in CFU fold change in both conditions. At all time points, there were no statistically significant differences ($p > 0.05$) between cells in acidic versus neutral media, and the corresponding effect sizes ($r = 0.20–0.41$) indicated only small to moderate effects.

Acidity protects S. cerevisiae cells against oxidative stress

To examine the protective role of acidity under oxidative stress, cells grown in either acidic or regular media were exposed to 8 mM H_2O_2 for 30 minutes and analyzed for viability and ROS accumulation (Figure 2).

To assess viability, CFU fold changes were calculated every 10 minutes post-exposure. In regular medium, CFU fold changes at 10, 20, and 30 minutes were 0.371±0.08, 0.081±0.03, and 0.031±0.03, respectively. In acidic medium, corresponding values were 0.55±0.08, 0.262±0.10, and 0.181±0.07 (Figure 2A). Although these differences were not statistically significant ($p > 0.05$), the effect sizes were notably large ($r = 0.6682$, 0.7144, and 0.8165, respectively), suggesting a biologically meaningful protective effect of acidity.

Intracellular ROS accumulation was measured using H_2DCFDA staining. Following 30-minute exposure to 8 mM H_2O_2, cells in acidic medium showed significantly less ROS accumulation than those in neutral medium (Figure 2B).

A statistically significant difference was observed among groups ($KW = 10.20$, $p = 0.0169$). The percentage of DCF-positive cells was 18.35±3.49% in regular medium with H_2O_2, compared to

2.44±0.47% in acidic medium. While the pairwise difference did not reach significance ($p > 0.05$), the large effect size ($r = 0.8018$) suggests a substantial biological effect.

Acidic conditions increase autophagic activity in S. cerevisiae

To evaluate the impact of extracellular acidity on autophagy, cells were incubated in acidic or regular medium for 30 minutes and stained with MDC to assess autophagolysosome formation (Figure 3). A statistically significant difference in MDC fluorescence was detected between groups ($KW = 8.64$, $p = 0.0078$).

In the absence of H_2O_2, cells in acidic medium had higher MDC fluorescence (120.46±6.24) compared to neutral pH (97.68±6.05), although the difference was not statistically significant ($p > 0.05$). However, the effect size was large ($r = 0.8018$), indicating enhanced autophagic activity.

Under H_2O_2 stress, MDC fluorescence remained similar in neutral pH (97.39±8.98; $r = 0.0891$), but increased in acidic medium (115.48±5.35). While again not statistically significant, the moderate effect size ($r = 0.4454$) supports a role for acidity in enhancing autophagy under oxidative stress.

Extracellular acidity reduces apoptosis under oxidative stress

To test whether acidity reduces apoptosis induced by oxidative stress, cells were exposed to 8 mM H_2O_2 in either acidic or neutral medium for 30 minutes, and apoptotic ratios were measured using dual staining (Figure 4). A statistically significant difference was found across groups ($KW = 9.585$, $p = 0.0224$).

In the absence of H_2O_2, apoptosis was low in both acidic (0.33±0.02%) and neutral (0.16±0.02%) media, with no significant difference ($p > 0.05$, $r = 0.1782$). Following H_2O_2 exposure, the apoptotic ratio in neutral medium rose sharply to 21.06±2.15%, while in acidic medium it was significantly lower at 12.45±1.45%. Although the difference did not reach significance ($p > 0.05$), the effect size was large ($r = 0.8018$), suggesting that extracellular acidity reduces apoptosis under oxidative stress.

Apoptotic cell death occurs under both pH conditions, while autophagy-dependent death is observed only in acidic medium

To elucidate the pathways of cell death under both pH conditions, CFU fold changes were compared in WT and mutant strains lacking key apoptotic (yca1Δ, aif1Δ) or autophagic (atg4Δ, atg8Δ) genes, following 30-minute exposure to 8 mM H_2O_2 (Figure 5).

In neutral medium, CFU fold changes were as follows: WT (0.04±0.02), yca1Δ (0.095±0.02), aif1Δ (0.046±0.01), atg4Δ (0.502±0.01), and atg8Δ (0.068±0.01) (Figure 5A). No statistically significant differences were found, but the large effect size between WT and yca1Δ ($r = 0.6682$) suggests a functional role for Yca1-mediated apoptosis.

Aif1's role appeared limited, with a small effect size ($r = 0.2673$). Atg4Δ and atg8Δ mutants showed slightly higher viability, though the effect sizes were small to moderate ($r = 0.2673$ and 0.4454, respectively).

In acidic medium, CFU fold changes were: WT (0.103±0.01), yca1Δ (0.172±0.03), aif1Δ (0.118±0.03), atg4Δ (0.215±0.06), and atg8Δ (0.153±0.01) (Figure 5B). While differences with WT were not statistically significant, the large effect size for yca1Δ ($r = 0.8018$) suggests that Yca1 also plays a role in cell death under acidic conditions.

Aif1 again showed a small effect ($r = 0.2673$). Notably, atg4Δ and atg8Δ mutants exhibited markedly higher survival, with large effect sizes ($r = 0.8018$ and 0.6634, respectively), indicating that autophagy-dependent cell death occurs under acidic conditions.

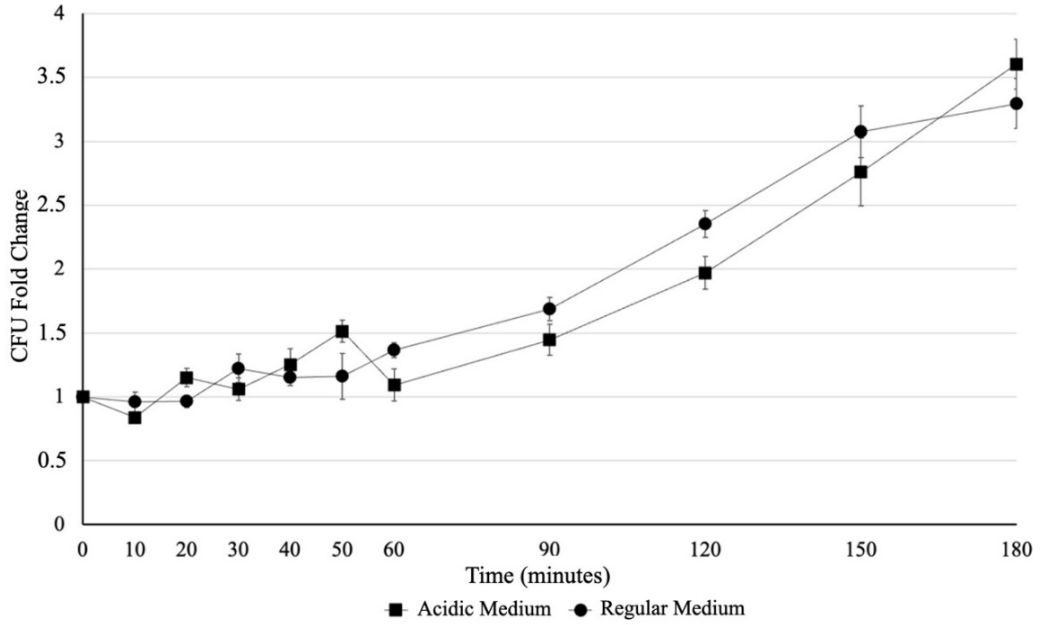

Figure 1. CFU fold changes of *S. cerevisiae* cells incubated in acidic and normal YPD media.

Data represent the mean and SD of three independent experiments. No statistically significant differences were observed between CFU fold change values at any sampling time between the acidic and regular pH media.

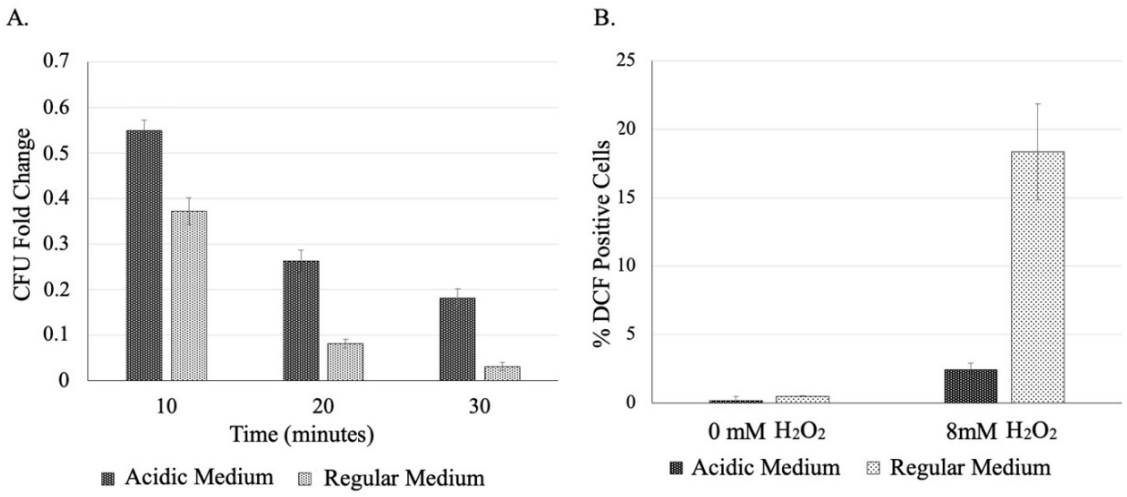

Figure 2. Protective effect of acidic environment against oxidative stress in *S. cerevisiae* cells.

A. CFU fold changes at 10, 20, and 30 minutes in cultures exposed to 8 mM H_2O_2 for 30 minutes in acidic or normal media. Data represent the mean and SD of four independent experiments. At each sampling time, a significant increase in cell viability was observed in response to H_2O_2 exposure under acidic conditions. B. Percentage of DCF-positive cells after 30-minute incubation in acidic and normal media with or without 8 mM H_2O_2. Data represent the mean and SD of three independent experiments. H_2O_2 exposure under acidic conditions significantly reduced intracellular ROS accumulation.

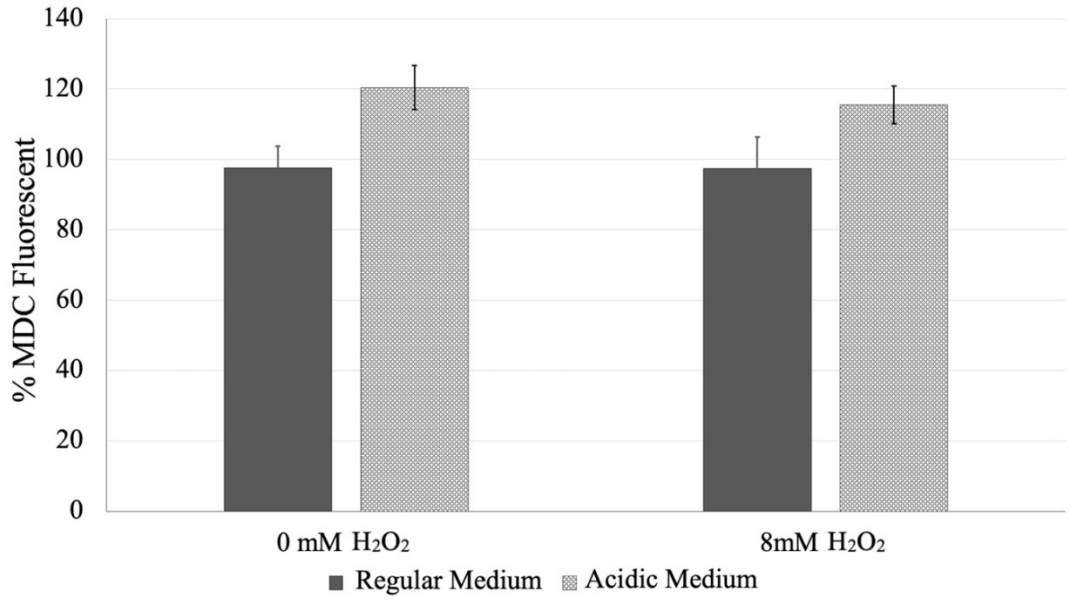

Figure 3. Effects of acidity on autophagic activity.

Percentage of MDC fluorescence in cells incubated for 30 minutes in acidic and regular media, with or without 8 mM H_2O_2. Data represent the mean and SD of three independent experiments. A significant increase in autophagic activity was observed in cells exposed to H_2O_2 under acidic conditions.

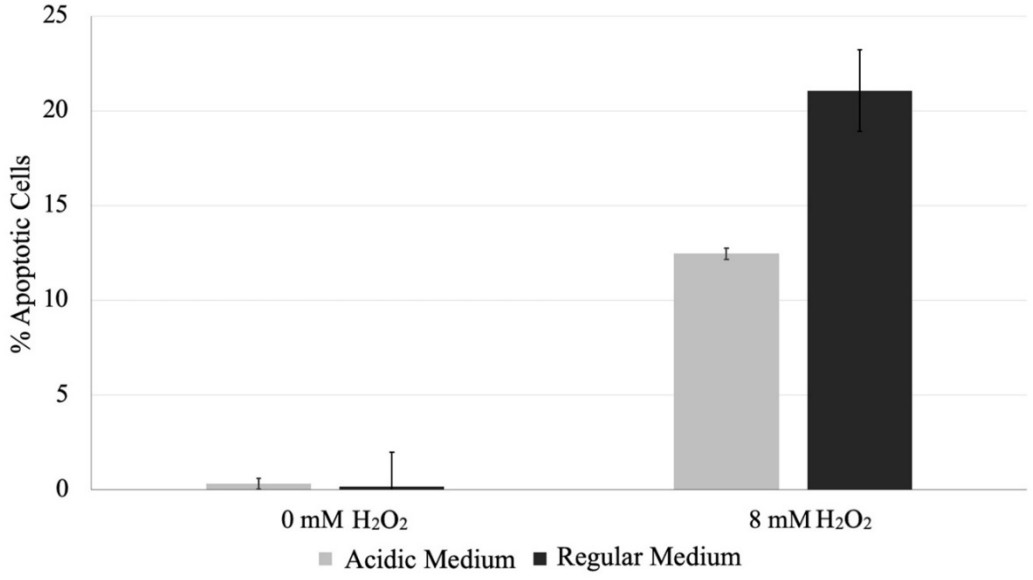

Figure 4. Effect of acidic environment on apoptotic cell ratio during oxidative stress-induced cell death. Percentage of apoptotic cells after 30-minute incubation in acidic and normal media with or without 8 mM H_2O_2. Data represent the mean and SD of three independent experiments. A significant reduction in the percentage of apoptotic cells was observed in H_2O_2-exposed cells under acidic conditions.

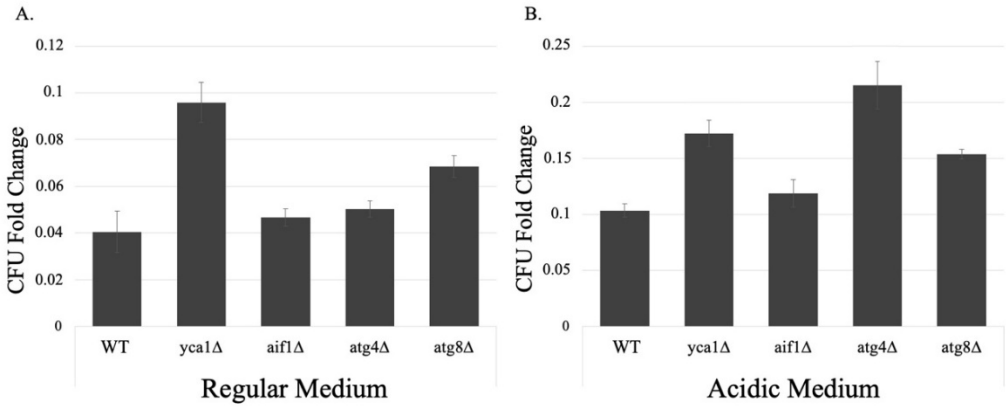

Figure 5. Effects of oxidative stress on cell death modes in *S. cerevisiae*.

A. CFU fold changes of wild-type (WT), *yca1*Δ, *aif1*Δ, *atg4*Δ, and *atg8*Δ *S. cerevisiae* strains after 30-minute exposure to 8 mM H_2O_2 in normal medium. Data represent the mean and SD of three independent experiments. Each mutant strain was compared to WT cells under the same conditions. B. CFU fold changes of WT, *yca1*Δ, *aif1*Δ, *atg4*Δ, and *atg8*Δ strains after 30-minute exposure to 8 mM H_2O_2 in acidic medium. Data represent the mean and SD of three independent experiments. Each mutant strain was compared to WT cells under acidic conditions.

DISCUSSION

In this study, we investigated the effects of extracellular acidity on resistance to H_2O_2-induced oxidative stress and its impact on the mode of cell death. We first examined whether acidic conditions alone had any effect on *S. cerevisiae* cells. Our findings showed that acidity alone did not impair yeast growth over a 3-hour incubation period, nor did it induce notable ROS accumulation or apoptosis, suggesting that a decrease in extracellular pH alone does not impose sufficient stress to compromise cell viability.

These results are consistent with previous findings. Malakar et al. reported that exposure of S. cerevisiae to moderately acidic conditions (pH ~3–4) for 2 hours had minimal impact on viability compared to controls[28]. This tolerance is attributed to the yeast's ability to maintain intracellular pH homeostasis by actively extruding excess protons[29]. However, at extremely low pH (~1), viability is dramatically reduced; in Malakar's study, almost all cells lost viability within 2 hours at pH ~1 [28]. In our study, the extracellular pH was adjusted to ~3.5, which aligns with conditions shown to be non-lethal to yeast in prior research. Although viability remained unaffected, acidic conditions significantly activated autophagy, even in the absence of oxidative stress. This is in line with findings from cancer research, where acidic microenvironments promote autophagy as a survival mechanism, such as in small cell lung cancer cells[20]. Pellegrini et al. also demonstrated that cancer cells engage autophagy as a protective response to acidic stress [21].

After establishing that extracellular acidity does not independently induce cell death, we investigated its role in modulating responses to oxidative stress. Our data indicate that acidic conditions conferred a protective effect against H_2O_2-induced oxidative damage. Supporting this observation, earlier studies have shown that brief pre-incubation in acidic medium renders *S. cerevisiae* resistant to subsequent acetic acid-induced death [18,19]. Although our approach applied acidic pH and oxidative stress concurrently (rather than as preconditioning), the protective effect observed may similarly involve enhanced antioxidant responses, such as upregulation of catalase and superoxide dismutase activities, as proposed in those studies. This mechanism may explain the lower intracellular ROS levels observed in our acidic conditions, reinforcing the conclusion that extracellular acidity mitigates oxidative damage, in agreement with our viability data.

The observed reduction in apoptosis may be attributed to either diminished ROS levels or to acidity-induced autophagy, which occurred even without oxidative stress in our study. Autophagy is a

conserved self-degradation pathway that removes damaged proteins, organelles, and intracellular pathogens to preserve cell homeostasis[30,31]. Its protective role likely involves both the clearance of ROS-damaged cellular structures and suppression of apoptosis. For example, resistin-induced autophagy has been shown to protect breast cancer cells from doxorubicin-induced apoptosis[32]. Similarly, increased autophagy under acidic conditions may contribute to decreased apoptotic cell death, a finding also supported by studies in Jurkat cells, where acidic media reduced doxorubicin-induced apoptosis [33].

In *S. cerevisiae*, Yca1 and Aif1 are involved in caspase-dependent and -independent apoptosis, respectively, while Atg4 and Atg8 are essential for autophagy. Consistent with this, deletion of these genes (*yca1⊿, aif1⊿, atg4⊿, atg8⊿*) allowed us to dissect the role of each pathway. The enhanced survival of yca1Δ strains in both pH conditions suggests that caspase-dependent apoptosis contributes to oxidative stress-induced cell death. Meanwhile, the markedly higher survival of *atg4⊿* and *atg8⊿* strains under acidic conditions indicates that autophagy-dependent cell death occurs selectively in acidic environments. In contrast, autophagy gene deletions had little effect on survival under neutral conditions, suggesting that autophagy does not contribute significantly to cell death in such environments. This points to a dual role for autophagy in acidic conditions—as both a survival mechanism and a contributor to cell death, depending on cellular context and stress intensity.

While these findings are insightful, several limitations should be acknowledged. Only a single acidic pH (3.5) and a single H_2O_2 concentration (8 mM) were used. Although these parameters were selected based on prior optimization, they limit our ability to assess dose-dependent responses or pH thresholds. Furthermore, some limitations stem from the specificity of staining assays used. MDC, although widely utilized, is not entirely specific to autophagosomes and may label other acidic compartments. Hoechst/PI staining, while useful for identifying apoptosis and necrosis, may not detect early apoptotic events. H_2DCFDA, though commonly used for ROS detection, is primarily sensitive to H_2O_2 and may not fully capture other reactive oxygen species. Although our short exposure window (30 minutes) aligns with the yeast cell doubling time (~90 minutes) and is suitable for acute stress analysis, longer exposures might reveal adaptive responses or cell cycle-dependent effects, which were beyond the scope of this work. Nonetheless, the use of multiple complementary assays, combined with mutant strains deficient in key apoptotic and autophagic regulators, strengthens the validity of our findings. Still, as our work is based on *S. cerevisiae*, a unicellular model, caution should be taken when extrapolating to multicellular systems, despite the conservation of many stress response pathways.

In conclusion, this study investigated the impact of extracellular acidity on oxidative stress resistance and the regulation of cell death pathways in *Saccharomyces cerevisiae*. Our findings demonstrate that acidic conditions attenuate intracellular ROS accumulation, suppress apoptosis, and enhance autophagic activity under oxidative stress. Notably, autophagy exhibited a dual role in acidic environments, functioning both as a protective mechanism and, under certain conditions, as a contributor to cell death. These results indicate that extracellular pH modulates the interplay between autophagy and apoptosis, ultimately shaping the cellular response to oxidative damage. Given the prevalence of acidic microenvironments in pathological contexts such as solid tumors and inflamed tissues, these insights may inform future therapeutic strategies aimed at manipulating pH-dependent stress responses to promote cell survival or sensitize cells to death signals.

Author Contributions: Concept/Design : NK; Data acquisition: NK, DK, PBT; Data analysis and interpretation: NK, PBT; Drafting manuscript: NK, DK; Critical revision of manuscript: NK, PBT; Final approval and accountability: NK, DK, PBT; Technical or material support: NK; Supervision: NK, PBT; Securing funding (if available): n/a.
Ethical Approval: This study does not require ethical approval as it does not involve human or animal subjects.
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REFERENCES

1. Kato Y, Ozawa S, Miyamoto C, Maehata Y, Suzuki A, Maeda T et al. Acidic extracellular microenvironment and cancer. Cancer Cell Int. 2013;13:89.
2. Smallbone K, Gavaghan DJ, Gatenby RA, Maini PK. The role of acidity in solid tumour growth and invasion. J Theor Biol. 2005;235:476–84.

3. da Silva VP, Mesquita CB, Nunes JS, de Bem Prunes B, Rados PV, Visioli F et al. Effects of extracellular acidity on resistance to chemotherapy treatment: a systematic review. Med Oncol. 2018;35:161.
4. Denk S, Neher MD, Messerer DAC, Wiegner R, Nilsson B, Rittirsch D et al. Complement C5a functions as a master switch for the pH balance in neutrophils exerting fundamental immunometabolic effects. J Immunol. 2017;198:4846–54.
5. Jiang W, Le J, Wang PY, Cheng X, Smelkinson M, Dong W et al. Extracellular acidity reprograms macrophage metabolism and innate responsiveness. J Immunol. 2021;206:3021–31.
6. Trevani AS, Andonegui G, Giordano M, López DH, Gamberale R, Minucci F et al. Extracellular acidification induces human neutrophil activation. J Immunol. 1999;162:4849–57.
7. Chen A, Dong L, Leffler NR, Asch AS, Witte ON, Yang LV. Activation of GPR4 by acidosis increases endothelial cell adhesion through the cAMP/Epac pathway. PLoS One. 2011;6:e27586.
8. D'Arcangelo D, Facchiano F, Barlucchi LM, Melillo G, Illi B, Testolin L et al. Acidosis inhibits endothelial cell apoptosis and function and induces basic fibroblast growth factor and vascular endothelial growth factor expression. Circ Res. 2000;86:312–8.
9. Díaz FE, Dantas E, Cabrera M, Benítez CA, Delpino MV, Duette G et al. Fever-range hyperthermia improves the anti-apoptotic effect induced by low pH on human neutrophils promoting a proangiogenic profile. Cell Death Dis. 2016;7:e2437.
10. Recek N, Zhou R, Zhou R, Te'o VSJ, Speight RE, Mozetič M et al. Improved fermentation efficiency of S. cerevisiae by changing glycolytic metabolic pathways with plasma agitation. Sci Rep. 2018;8:8252.
11. Farrugia G, Balzan R. Oxidative stress and programmed cell death in yeast. Front Oncol. 2012;2:64.
12. Madeo F, Carmona-Gutierrez D, Ring J, Büttner S, Eisenberg T, Kroemer G. Caspase-dependent and caspase-independent cell death pathways in yeast. Biochem Biophys Res Commun. 2009;382:227–31.
13. Falcone C, Mazzoni C. External and internal triggers of cell death in yeast. Cell Mol Life Sci. 2016;73:2237–50.
14. Anding AL, Baehrecke EH. Cleaning house: selective autophagy of organelles. Dev Cell. 2017;41:10–22.
15. Gozuacik D, Kimchi A. Autophagy and cell death. Curr Top Dev Biol. 2007;78:217–45.
16. Boya P, González-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N et al. Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol. 2005;25:1025–40
17. Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG et al. Remodeling of yeast genome expression in response to environmental changes. Mol Biol Cell. 2001;12:323–37.
18. Guaragnella N, Stirpe M, Marzulli D, Mazzoni C, Giannattasio S. Acid stress triggers resistance to acetic acid-induced regulated cell death through Hog1 activation which requires RTG2 in yeast. Oxid Med Cell Longev. 2019;2019:4651062.
19. Giannattasio S, Guaragnella N, Corte-Real M, Passarella S, Marra E. Acid stress adaptation protects Saccharomyces cerevisiae from acetic acid-induced programmed cell death. Gene. 2005;354:93–8.
20. Xie WY, Zhou XD, Li Q, Chen LX, Ran DH. Acid-induced autophagy protects human lung cancer cells from apoptosis by activating ER stress. Exp Cell Res. 2015;339:270–9.
21. Pellegrini P, Strambi A, Zipoli C, Hägg-Olofsson M, Buoncervello M, Linder S et al. Acidic extracellular pH neutralizes the autophagy-inhibiting activity of chloroquine: implications for cancer therapies. Autophagy. 2014;10:562–71.
22. Atalay PB, Kaluc N, Cavusoglu EE. Effect of mitosis on the resistance to oxidative and osmotic stresses in yeast. Sakarya University Journal of Science. 2020;24:265–71.
23. Atalay PB, Kaluç N. Mitoz sırasında H_2O_2 ile indüklenen oksidatif strese karşı dirençte Yca1'in rolünün incelenmesi. Mersin Üniversitesi Sağlık Bilimleri Dergisi. 2019;12:230–39.
24. Chen X, Zhong Z, Xu Z, Chen L, Wang Y. 2',7'-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: forty years of application and controversy. Free Radic Res. 2010;44:587–604.
25. Kaluç N, Thomas PB. Hypochlorous acid induces caspase dependent apoptosis in Saccharomyces cerevisiae. Journal of Biosciences and Medicines. 2021;9:42–53.
26. Munafó DB, Colombo MI. A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci. 2001;114:3619–29.
27. Biederbick A, Kern HF, Elsässer HP. Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur J Cell Biol. 1995;66:3–14.
28. Malakar D, Dey A, Ghosh AK. Protective role of S-adenosyl-L-methionine against hydrochloric acid stress in Saccharomyces cerevisiae. Biochim Biophys Acta. 2006;1760:1298–303.
29. de Melo HF, Bonini BM, Thevelein J, Simões DA, Morais MA Jr. Physiological and molecular analysis of the stress response of Saccharomyces cerevisiae imposed by strong inorganic acid with implication to industrial fermentations. J Appl Microbiol. 2010;109:116–27.
30. Wei F, Xie Q, Huang Z, Yang A, Duan Y. Induction of autophagy and endoplasmic reticulum autophagy caused by cadmium telluride quantum dots are protective mechanisms of yeast cell. J Appl Toxicol. 2022;42:1146–58.

31. Li W, He P, Huang Y, Li YF, Lu J, Li M et al. Selective autophagy of intracellular organelles: recent research advances. Theranostics. 2021;11:222–56.
32. Liu Z, Shi A, Song D, Han B, Zhang Z, Ma L et al. Resistin confers resistance to doxorubicin-induced apoptosis in human breast cancer cells through autophagy induction. Am J Cancer Res. 2017;7:574–83.
33. Bohloli M, Atashi A, Soleimani M, Kaviani S, Anbarlou A. Investigating effects of acidic pH on proliferation, invasion and drug-induced apoptosis in lymphoblastic leukemia. Cancer Microenviron. 2016;9:119–26.